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THE MODE OF EXCRETION OF AMMONIA AND UREA IN *XENOPUS LAEVIS*

BY J. B. BALINSKY* AND E. BALDWIN
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(*Received* 10 *May* 1961)

INTRODUCTION

Munro (1953) showed that the main nitrogenous excretory products of *Xenopus laevis* are ammonia and urea, the former compound predominating over the latter. The work of Underhay & Baldwin (1955) and of Balinsky, Cragg & Baldwin (1961) suggests that the relative amounts of ammonia and urea in the urine of *Xenopus* are not always the same, but are dependent on the stage of development of the animals and also on the environment. In order to find out what determines the amount of each waste product excreted, it was considered important to investigate the mode of excretion of each.

Urea excretion in typical frogs such as *Rana* species differs from the process in mammals, since it involves active tubular secretion. This is indicated by the fact that the urine/plasma ratio of urea in *Rana catesbeiana*, unlike that of mammals, is dependent on blood urea concentration (Marshall & Crane, 1924). At high concentration, the ratio decreases, so that urea excretion tends to a maximum. Further evidence of tubular secretion is provided by the fact that urea clearance is greater than creatinine clearance in *R. clamitans* (Schmidt-Nielsen & Forster, 1954), and that treatment with phlorhizin greatly decreases the urine/plasma ratio of urea in *R. pipiens* (Walker & Hudson, 1937).

Ammonia excretion in *Rana* occurs by active secretion in the distal tubules and collecting ducts of the kidney (Walker, 1940). It seems to be formed there, as the blood ammonia level in *R. temporaria* is apparently zero (Florkin, 1943). It should be borne in mind, however, that, compared to *Xenopus*, *Rana* species excrete very little ammonia. The experiments of Przylecki (1925) with acidotic *R. esculenta* suggest that ammonia excretion in these animals serves the purpose of cation conservation, just as it does in mammals. Ammonia in *Xenopus* is primarily a nitrogenous waste product, and its mode of excretion need not necessarily be similar to the process in *Rana*.

The results of Przylecki, Opienska & Giedroyc (1922) suggest that the skin plays some part in the excretion of nitrogenous waste products, though not more than 15% if the waste nitrogen is excreted via the skin at 22° C. The possibility of skin excretion in *Xenopus* was tested in the present work.

A preliminary report of the present work has already been published (Balinsky, 1960).

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MATERIALS AND METHODS

Experimental animals. The animals used in the present investigation were male specimens of *Xenopus laevis*, recently imported from South Africa, and weighing 40–60 g. They were kept in water, in glass aquaria which were cleaned out frequently, and fed once weekly on chopped liver.

Collection of urine. Experiments were normally started on the day after the animals had been fed. Immediately prior to the first urine collection of each experiment, each animal was weighed. This weight was used for all calculations relating to the experiment. During the periods of urine collection, the animals were kept in a constant temperature room at 20° C. to prevent variation in excretion due to temperature changes (see below under Results). Urine was collected by one of the following methods (unless otherwise stated, Method 1 was used).

(1) Each animal was kept separately for a 24 hr. period in 150 ml. of distilled water in a 1 l. beaker, covered with a glass sheet. The urine was voided into the surrounding fluid.

Preliminary experiments indicated that, when urine is collected in this way, there is a possibility of bacterial decomposition of urea. They also indicated that no decomposition occurred for at least 48 hr. if the fluid contained chloramphenicol (0·001 %), streptomycin (0·001 %), or terramycin (0·0001 %). Therefore, a few drops of 0·2 % terramycin (intramuscular, Chas. Pfizer and Co. Inc., New York) were added to each beaker used for urine collection.

After the collection period, the animal and its surrounding fluid were poured into a Buchner funnel, the fluid draining into a flask below. The animal was lightly washed, the washings likewise draining into the collection flask. The fluid collected was centrifuged if necessary, to remove solid debris. It was acidified to pH 1–2 with a little sulphuric acid, and made up to a suitable volume.

The above method does not give any information on the volume of urine produced by the animal. In order to determine this, and thus to know the concentrations of waste products in the urine, one of the following methods was used.

(2) *Cloacal ligature.* Each animal was anaesthetized by placing in a 0·3 % solution of metacain (M.S. 222, from Sandoz Ltd., Basle, Switzerland). A stitch was made with a surgical needle, passing a loop of cotton around the cloaca, as described by Ewer (1950). The cloaca was then ligatured by tying this loop into a knot. The animals were allowed to recover for 30 min. in running water and placed in a beaker with some water. After about 6 hr. the animals were killed and their urinary bladders were emptied. The volume of urine thus collected was noted. The samples were diluted somewhat, centrifuged, acidified, and made up to volume as before.

(3) *Cloacal cannulation* (Ewer, 1950). The procedure was similar to that in the previous method, except that, when the stitch around the cloaca had been made, a small glass cannula, attached to a toy rubber balloon, was introduced into the cloaca and fixed by ligaturing as described by Ewer (1950). The balloon contained a few drops of 0·2 % terramycin. After a 24 hr. collection period, the stitch was opened and the cannula with its balloon was removed. The urine was collected and its volume was noted. It was centrifuged, acidified, and made up to volume.

The advantage of the last method is that, during the 24 hr. collection period, the animal can be kept in a beaker as under Method 1, so that any waste products excreted otherwise than through the cloaca can be collected and assayed separately.

Urine analysis. Aliquots of the urine samples obtained as described above were used for assay of urea and ammonia as follows:

Urea. The colorimetric method of Archibald (1945) was used, except that 0·5 ml. of 0·015 M manganese sulphate were added to each tube. This intensified the colour due to urea, and appeared to make it more reproducible. The α-isonitroso-propiophenone was prepared from propiophenone by the method of Claisen & Manasse (1889).

Ammonia. The procedure adopted was based on Russell's (1944) colorimetric method, but with certain modifications suggested by experience.

Underhay & Baldwin (1955) had found that the preparation of potassium hypochlorite suggested by Russell could be obviated by using a commercial hypochlorite solution. In addition, the present authors found that alkaline phenol on storage invariably underwent slight oxidation, and this caused marked inhibition of the colour formed by Russell's method. The following modified method was therefore used.

Reagents

Sodium hypochlorite, containing 1·3–1·4% free chlorine was prepared by dilution of commercial sodium hypochlorite (B.D.H. Ltd., 10–13% free chlorine), and standardized as described by Russell (1944).

Phenol. To 10 ml. of 10^{-2} M potassium cyanide were added 90 g. of phenol. From the resulting solution, phenol crystallized out on storage in the refrigerator, but it could be redissolved on gentle warming. The solution remained colourless for months.

Sodium hydroxide, 23%.

Manganese sulphate, 4×10^{-3} M.

All solutions were stored in brown bottles in the refrigerator, and were stable for months.

Procedure

Immediately before use, 3 vol. of the phenol solution were mixed with 5 vol. of the sodium hydroxide in an ice bath. The resulting solution was used within 15 min. of preparation.

5 ml. samples containing ammonia to be determined were placed in graduated tubes standing in an ice bath. Two drops of manganese sulphate solution, 0·8 ml. of the phenol–sodium hydroxide solution, and 1 ml. of sodium hypochlorite solution were added to the tubes in the above order. The tubes were covered with sheets of cellophane, and vigorously shaken. They were incubated in a boiling water-bath for 5 min., rapidly cooled, and again vigorously shaken. The colours were read in a colorimeter using an Ilford 204 filter.

Blood analysis. Blood was obtained from freshly killed frogs by cutting the truncus arteriosus, and collecting the blood in a tube containing a few crystals of sodium citrate. Samples of blood were then used for determining the concentrations of urea and ammonia.

Urea. The blood was deproteinized by addition of an approximately equal volume of 20% trichloroacetic acid. After dilution with water, the precipitated protein was removed by centrifuging, and the sample was made up to a suitable volume. Aliquots of this were analysed for urea by Archibald's method as described above.

Ammonia. Conway's microdiffusion technique was used in conjunction with the colorimetric method described above. The inner chamber of each standard microdiffusion unit (Conway & Byrne, 1933) contained 1 ml. of 0·1 N-hydrochloric acid. Into the outer chamber, containing 1 ml. of saturated potassium carbonate, were pipetted 0·3 ml. of blood at a measured time after shedding. After diffusion had proceeded for 2 hr. at room temperature, the contents of the inner chamber were transferred to a graduated tube by means of a fine pipette with a rubber teat. The contents of the inner chamber were washed with four portions of 1 ml. of distilled water, and the washings were also poured into the graduated tube. The contents of the tube were analysed for ammonia as described above.

Table 1. *Nitrogen excretion of* Xenopus laevis *on the first day after feeding*

	Nitrogen excreted (μg./g. body wt./24 hr.)			Ammonia N as percentage of urea N +ammonia N
	Urea N	Ammonia N	Urea N +ammonia N	
Means of 82 experiments	107·3	165·2	272·5	62·23
Standard errors of means	±8·60	±7·51	±13·76	±1·320
Standard deviations	77·9	68·0	124·6	11·95
Coefficients of variation (standard deviations as percentages of means)	72·6	41·2	45·7	31·6

RESULTS

Variability of excretion. Eighty-two single determinations of ammonia and urea excretion of *Xenopus* on the day after feeding were made. The results are summarized in Table 1. A great variability (from 40 to 80%) was found in the percentage of ammonia nitrogen among the waste products. A consideration of the coefficients of variation (standard deviations as percentages of mean values) of the actual amounts of ammonia and urea excreted indicates that there was relatively greater variability in the amount of urea than in the amount of ammonia excreted. The mean percentage of ammonia nitrogen was rather lower than that reported by Munro (1953). This may have been the result of precautions taken in the present work to avoid bacterial decomposition of urea to ammonia.

The nitrogen excretion of the same animals on successive days was then determined. Fig. 1 A shows the urea excretion of fifteen animals on 3 successive days after feeding. It will be seen that the excretion of ammonia declined more or less steadily over the 3 days, while the urea excretion decreased more slowly. As a result of this, more nitrogen was excreted as urea than as ammonia on the third day.

Fig. 1 B shows the means of five experiments carried out over 11 days, the animals receiving no food for the duration of the experiments. Though the mean urea output of this batch of animals was initially lower, the same trend as in Fig. 1 A can be observed. Compared to the first day, there was a large decline of ammonia production by the fourth day, but no decline of urea production. On subsequent days, both ammonia and urea excretion decreased.

Fig. 1C represents the effect of prolonged starvation on the nitrogen excretion of *Xenopus*. It shows the means of two experiments. Once again, a great decrease of ammonia excretion after the first day was observed. By the fifteenth day the animals appeared to have reached a starvation level of excretion, which showed no further pronounced changes.

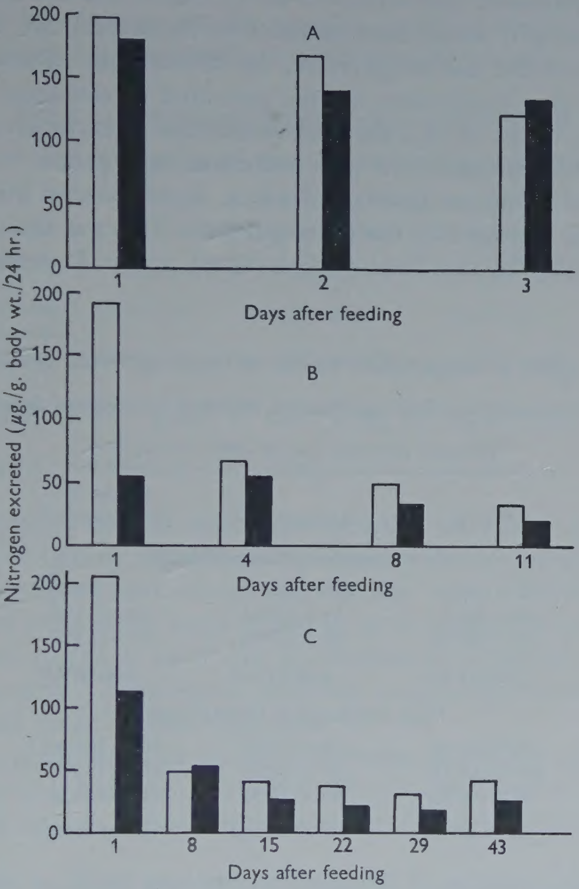

Fig. 1. Nitrogen excretion of *Xenopus laevis* on successive days after feeding. A, over 3 days, means of fifteen experiments; B, over 11 days, means of five experiments; C, over 43 days, means of two experiments. White areas: ammonia nitrogen excreted; black areas: urea nitrogen excreted.

It can be concluded that *X. laevis* excretes large amounts of ammonia after feeding, but the ammonia excretion soon declines. The urea excretion continues at a more or less constant level for the first 3 or 4 days after feeding, often exceeding the ammonia nitrogen output on about the third day. The outputs of the two waste products are thus to some degree independent of each other.

The effect of temperature. The effect of temperature on nitrogen excretion was next examined. To eliminate error due to individual variation, it was necessary to obtain separate results for each individual animal. The following procedure was therefore adopted. On the first day the animals were placed in beakers for urine collection at the usual temperature (20° C.). On the following morning their urine was collected and the animals were placed in an incubator regulated at the test temperature, either 30 or 10° C. After some hours they were again placed in beakers for urine collection overnight. On the third day they were returned to the original temperature and, after some hours, they were once more placed in beakers for overnight urine collection. A mean of the excretions of each animal at 20° C. on the first and third days was used as an estimate of what it would have excreted on the second day, and compared to its actual excretion at the test temperature. By dividing the measured or estimated excretion at the higher temperature by the measured or estimated excretion at the lower temperature, values of Q_{10}, the relative increase in excretion per 10° C. rise in temperature, could be calculated for each individual experiment.

The means of the results are shown in Table 2. It will be seen that nitrogen excretion in *X. laevis* was increased by rise in temperature. This was also observed in *Rana pipiens* by van der Heyde (1921) and Adolph (1927), and in *R. esculenta* by Przylecki *et al.* (1922).

Table 2. *The effect of temperature on the nitrogen excretion of* Xenopus laevis

The figures quoted are means of four experiments, followed by standard deviations in brackets.

		Nitrogen excreted (μg./g. body wt./24 hr.)			Ammonia N as percentage of urea N + ammonia N
	Temp. (°C.)	Urea N	Ammonia N	Urea N + ammonia N	
		Low temperature experiments			
1st day	20	80·0 (14·4)	158·3 (70·4)	238·3 (82·3)	64·38 (9·68)
2nd day	10	26·0 (20·5)	31·3 (20·2)	57·3 (44·4)	56·95 (6·79)
3rd day	20	79·3 (35·9)	98·3 (32·6)	177·6 (54·8)	55·90 (9·79)
Mean Q_{10}'s		5·05 (2·21)	4·78 (3·14)	4·90 (2·63)	
		High temperature experiments			
1st day	20	58·8 (23·7)	130·5 (38·5)	189·3 (57·2)	69·38 (9·76)
2nd day	30	130·8 (48·7)	177·5 (42·6)	308·3 (73·8)	58·33 (8·97)
3rd day	20	50·3 (19·8)	60·5 (17·5)	110·8 (20·9)	55·33 (15·50)
Mean Q_{10}'s		2·50 (0·61)	1·88 (0·23)	2·07 (0·23)	

Ammonia and urea excretion are apparently equally affected. It is thus possible that variation in waste nitrogen output merely reflects variation in the volume of urine produced by the animals at different temperatures.

Between 20 and 30° C. the Q_{10} values for ammonia and urea were near 2. Cooling the animals from 20 to 10° C., however, produced a more than twofold decrease in ammonia and urea excretion. This finding is similar to that of Przylecki *et al.* (1922), though at variance with those of van der Heyde (1921) and Adolph (1927).

Cutaneous excretion. It was considered possible that the skin of *Xenopus laevis*, being constantly in contact with water, might serve as an important organ of nitrogen excretion. Cutaneous excretion might even explain the ammoniotelism of *Xenopus*, or the partial independence of its two waste products.

The possibility of excretion through the skin was tested by cloacal cannulation, and simultaneous determination of excretion by Method 1. Preliminary experiments suggested that nitrogen excretion was not affected by cannulation.

The results of the experiments are shown in Table 3. It will be seen that, on the average, at least 86% of ammonia and 81% of urea, constituting some 85% of the total nitrogen, were excreted through the cloaca. This result corresponds to that of Przylecki *et al.* (1922) for *Rana esculenta* (85·5% of non-protein nitrogen at 22° C.).

The significance of the remaining 15% is not clear. It might represent leakage around the cannula rather than skin excretion. In any event, it seems that *Xenopus* does not make greater use of its skin for nitrogen excretion than do other frogs. Nor is any one waste product preferentially excreted through the skin.

Table 3. *Cutaneous excretion in* Xenopus laevis

The figures quoted are means of fourteen experiments, followed by standard deviations in brackets.

	Nitrogen excreted (μg./g. body wt./24 hr.)			Ammonia N as percentage of urea N +ammonia N
	Urea N	Ammonia N	Urea N +ammonia N	
Collected in rubber balloon attached to cloacal cannula	46·7 (21·0)	103·1 (41·2)	149·8 (57·6)	68·69 (7·39)
Collected in surrounding fluid	10·6 (5·7)	16·8 (10·3)	27·4 (15·0)	58·71 (15·49)
Mean percentage of total collected in balloon	81·1 (7·20)	86·0 (7·69)	84·4 (6·96)	—
Standard errors of means	± 1·93	± 2·05	± 1·86	—

The urine of Xenopus. Cloacal cannulation provided samples of undiluted *Xenopus* urine. The mean of twenty determinations of urine volume produced in 24 hr. at 20° C. was 23·6 ± 1·19 ml. per 100 g. body weight, with a standard deviation of 5·33 ml. A value of 31% of the body weight at 20° C. is quoted for *Rana pipiens* by Adolph (1927), and 16·5% at 15° C. for *R. esculenta* by Przylecki (1922). It seems that the urine output of *Xenopus* given abundant water supply is very comparable to that of *Rana* species under similar conditions.

The urine had a pH of 8·0, and was thus decidedly alkaline, in contrast to the acid urine (pH 4·6–7·0) produced by *R. pipiens* (Montgomery & Pierce, 1937). This is probably caused by a much higher concentration of ammonia in the urine of *Xenopus*.

Table 4. *Blood ammonia concentration in* Xenopus laevis

The figures quoted are means of three experiments.

Expts nos.	Time after shedding (sec.)	Ammonia concentration (μg. N/ml.)
1–3	55	8·13
	130	8·10
	255	8·90
4–6	15–19	5·57
Standard deviation		0·33

Comparison of blood and urinary concentrations of nitrogenous waste products. The results of blood ammonia measurements in *Xenopus* are shown in Table 4. *Xenopus* differs from *Rana temporaria* (Florkin, 1943) in that no rapid increase of blood ammonia after shedding could be observed (Expts. 1–3). In later experiments

(4–6), ammonia determinations were made as rapidly after shedding as possible. The results were only slightly lower than in the previous experiments. It seems, therefore, either that the true blood ammonia concentration in *Xenopus* is not zero, or else that the initial increase in concentration is extremely rapid, being over in less than 20 sec. The mean of the last three experiments was taken as the maximum blood ammonia concentration. Urinary urea concentration was also measured. A comparison of blood and urinary concentration of the two waste products is shown in Table 5. It will be seen that the urinary urea concentration was almost equal to that of blood urea. The urine/blood ratio was slightly higher than 1, but the difference is not significant at the 5% level. Urinary ammonia concentration, by contrast, was more than a hundred times greater than the corresponding value in the blood. It can be concluded that the mechanisms of excretion of ammonia and urea are very different.

Table 5. *Comparison of blood and urinary concentrations of ammonia and urea in* Xenopus laevis

The figures quoted are mean values, followed by standard deviations in brackets.

Compound	No. of expts.	Concentrations (μg. N/ml.) Blood	Urine	Urine/blood concentration ratio
Ammonia	3	5·57 (0·33)	610 (478)	110
Urea	15	172·1 (122·9)	195·1 (151·1)	1·135 (0·267)
Standard errors of means		±31·8	±39·1	±0·069

A correlation of blood and urinary urea concentrations in individual animals was also made. Urine was collected either by cloacal cannulation (for 24 hr.), or by cloacal ligature (for 6 hr.). The animals were then killed, and blood samples were analysed for urea. In some experiments, blood urea was artificially raised by injection of 0·4–2·0 ml. of 6% urea solution into the subcutaneous lymph spaces. The urine was collected for 6 hr. by cloacal ligature. The results are shown in Fig. 2, urinary urea concentration being plotted against blood urea concentration. A logarithmic scale was chosen, in order to include a large range of concentrations.

An important source of error in these experiments was that blood urea was measured at the end, rather than in the middle of the urine collection period. This was inevitable, as the small size of the animals made it very difficult to collect a sufficiently large sample of blood from them without killing them. The error was least in experiments with a 6 hr. collection period, but greatest when urea had been injected and its level in the blood was rapidly changing.

The results of an experiment on dehydrated animals (Balinsky *et al.* 1961) is also shown. In this case, the above-mentioned error was avoided by the following procedure. Urinary urea concentration was measured in two animals during their first 24 hr. in water, after 14 days' dehydration. The mean of these two determinations is plotted against the mean of four blood urea determinations on animals from the same batch, two before re-immersion in water and two after 24 hr. in water.

A plot of the results of all these experiments indicates the variation of urinary urea with different levels of blood urea over a very large range of urea concentrations. While the points show a considerable amount of scatter, they indicate an unmistakably linear correlation. The solid fitted line is very close to the line of unit slope through the origin (broken line). It appears, therefore, that, over a wide range of concentrations, the urinary urea concentration is almost equal to the blood urea concentration. This confirms the conclusion drawn from the results shown in Table 5.

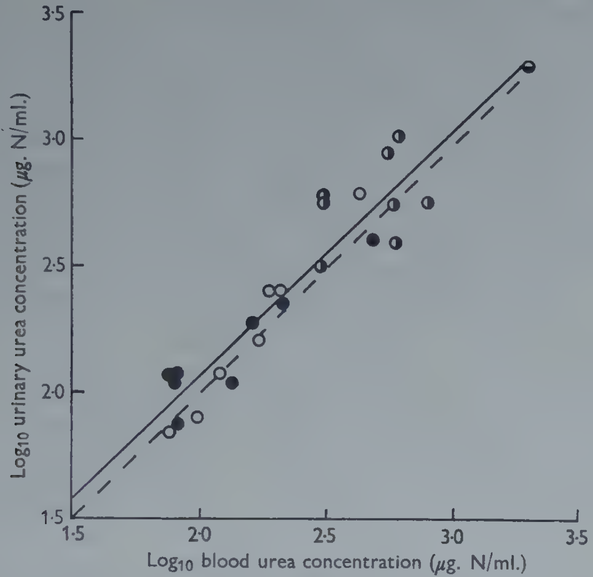

Fig. 2. Comparison of blood and urinary concentrations of urea in *Xenopus laevis*. ●, Urine collected by 24 hr. cloacal cannulation. ○, Urine collected by 6 hr. cloacal ligature. ◑, Urea injected subcutaneously, urine collected by 6 hr. cloacal cannulation. ◒, Urea accumulation induced by keeping out of water for 14 days, for details see text. Solid line: line fitted to the points by the method of least squares; broken line: line through the origin, of unit slope. (All urea concentrations on logarithmic scale.)

DISCUSSION

The experiments represented in Fig. 1 indicate a certain degree of independence of ammonia and urea excretion in *Xenopus laevis*. It seems that the two nitrogenous waste products are formed and excreted by two different, partly independent processes. Ammonia excretion greatly predominates during the first couple of days after feeding. Urea excretion is not as high, but remains at a steady level for a longer period of time before declining. It seems that ammonia is formed more rapidly, perhaps more directly, from the ingested amino acids. This results in a large output of ammonia after feeding, and appears to be the cause of ammoniotelism in *Xenopus*. It would be interesting to see whether the period of high ammonia excretion corresponds to the period of high concentration of ingested amino acids in the blood.

The results of the experiments described above should now be assessed for the light which they shed on the difference between ammonia and urea excretion in *X. laevis*. Both seem to be equally temperature-dependent. Neither occurs through the skin to any great extent.

A comparison of blood and urinary concentrations again emphasizes the difference between ammonia and urea excretion. While the urine/blood concentration ratio of ammonia is over a hundred, that of urea hardly exceeds unity. The latter result should be contrasted with the findings of Marshall & Crane (1924) in *Rana catesbeiana*, which indicate that the urine/plasma ratio of urea is always greater than 1, varying from 2 to 74 (Crane, 1927). Moreover, the value of this ratio is not constant, but decreases with increasing urea concentration. The latter fact, it will be remembered, is evidence for tubular secretion of urea in *R. catesbeiana*.

The present results resemble rather those of Addis & Drury (1923), that urea excretion in human subjects is proportional to blood urea concentration. It can be concluded that urea excretion in *Xenopus* resembles the process in mammals rather than in *Rana*, being brought about by glomerular filtration rather than active secretion.

It is interesting that, in *Xenopus laevis*, the blood/urine concentration ratio of urea is not only constant, but actually near to unity. This indicates that there is probably little or no reabsorption of water in the kidney tubules. This is an interesting result, as even in the kidney of the aquatic *Necturus*, which does not secrete urea actively, some water reabsorption is indicated (Walker & Hudson, 1937).

The absence of water reabsorption in *Xenopus* kidney indicates that the high urine/blood ratio of ammonia cannot be attributed to concentration of the urine subsequent to filtration. Ammonia must thus either be transported actively from blood to glomerular filtrate, or else actually formed in the cells lining the tubules. The former explanation can only be possible if the ammonia clearance, about 1100 ml./kg. hr. as calculated from the present results, does not exceed the renal plasma flow. The renal plasma flow in *Rana clamitans* is 775 ml./kg. hr. (Schmidt-Nielsen & Forster, 1954). No corresponding figure for *Xenopus* is available, and the possibility exists that it may be higher in that species. Even if this were so, complete clearance against a 100 to 1 concentration gradient would have to be postulated.

It is more likely that ammonia is formed in the kidney by the deamination of amino or amide nitrogen (Balinsky & Baldwin, 1961). This would imply that the mechanism of ammonia formation and excretion in *Xenopus* is similar to that in *Rana*, except that very much greater quantities of ammonia are excreted by the former.

In conclusion, it can thus be said that ammonia and urea excretion in *Xenopus* differ from each other because, in the excretion of urea, the kidney plays only a passive role, urea being made in the liver. Ammonia excretion, on the other hand, involves active tubular secretion and probably the formation of ammonia in the kidney.

SUMMARY

1. Eighty-two single determinations of ammonia and urea excretion by *Xenopus laevis* indicated that the percentage of ammonia varied from 40 to 80%, with a mean value of 62%.

2. Measurements of excretion on successive days after feeding showed that a large amount of ammonia was produced soon after feeding, but that ammonia excretion declined rapidly. Urea excretion, not so high initially, remained more or less constant until the third or fourth day, often exceeding ammonia excretion at that time. Thereafter, it also declined and the excretion of both substances reached a constant starvation level by the fifteenth day.

3. Both ammonia and urea excretion were equally affected by temperature. The Q_{10}'s were near 2 in the range 20–30° C., but greater in the range 10–20° C.

4. At least 86% of ammonia, and 81% of urea were excreted through the cloaca.

5. The mean 24 hr. urine output of *Xenopus* at 20° C. was 23·6 ml. per 100 g. body weight.

6. Although the blood ammonia concentration did not appear to be zero, the urine/blood concentration ratio of ammonia was greater than 100. The urine/blood concentration ratio of urea was not significantly different from unity, and constant over a very wide range of concentrations.

7. The above result is interpreted to indicate passive glomerular filtration of urea, and little or no tubular reabsorption of water.

8. It is suggested that ammonia is formed in the kidney, and actively secreted into the glomerular filtrate.

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STUDIES ON THE CHEMICAL COMPOSITION OF MUSCLE TISSUE

II. THE ABDOMINAL FLEXOR MUSCLES OF THE LOBSTER *NEPHROPS NORVEGICUS* (L.)

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I. INTRODUCTION

This is the second of a series of papers on the composition of muscles of some lower vertebrates and invertebrates, particularly from the standpoints of the osmotic concentration of various constituents and the accumulation or reduction of ions in muscle relative to those of the blood plasma. A comprehensive analysis of the muscle and plasma of the decapod crustacean *Nephrops norvegicus* (L.) has been made for all inorganic and organic compounds believed to be of osmotic significance. An attempt has been made to assign concentrations to the intracellular phase of muscle, after appropriate corrections for extracellular fluid, and to see how far the summation of ions and molecules compares with the experimentally determined osmotic concentration of the muscle and plasma.

Male specimens of *Nephrops* were obtained by trawling, and thereafter kept 3–4 days in the aquarium tanks at Millport. A chloride determination on the sea water was made each time an animal was used. Over the period of the analyses and experiments the values ranged between 17·76 and 18·80 g. Cl/l., with a mean of 18·4.

Blood was collected by pipette through the arthrodial membranes at the bases of the legs; after the cells had aggregated the blood was centrifuged and the clear plasma used for analysis. The muscles analysed were the paired longitudinal flexors of the abdomen. These were lightly blotted between sheets of filter paper and separate weighed samples were used for the determination of cations (sodium, potassium, calcium and magnesium), chloride, sulphate and water content. Methods used in these inorganic analyses of muscle and plasma have been given previously (Robertson, 1960*b*, *c*). In addition, further samples of muscle and plasma were analysed for other ions and compounds as follows: bicarbonate of muscle, method of Conway & Fearon (1944); lactate, method of Hullin & Noble (1953) on 10% trichloracetic acid filtrates of muscle and plasma; reducing sugar on zinc hydroxide filtrates after Somogyi (1945); inorganic and organic phosphate, fractionation procedure after Umbreit, Burris & Stauffer (1949), determination of phosphorus according to Sumner (1944); α-amino N of free amino acids on tungstic acid filtrates after Frame, Russell & Wilhelmi (1943) with the improvements of Russell (1944); arginine on tungstic acid filtrates by Macpherson's (1946) method, with the substitution of oxine for α-naphthol (Janus, 1956); proline according to Chinard (1952), after removing lysine with permutit (Troll & Lindsley, 1955); trimethylamine oxide and glycine betaine on 10% trichloracetic acid filtrates after Kermack, Lees & Wood (1955), with the substitution of gravimetric estimation of the mixed reineckates of these two compounds for colorimetry.

Estimates of total extracellular fluid in muscle, the blood plus interstitial fluid, were obtained by injecting into the sternal sinus solutions of inulin, sucrose or sodium thiosulphate, substances believed to enter cells only with difficulty, and comparing their distribution in samples of blood and muscle on a water-content basis, after periods of 1–10 hr. The volume or weight of water deemed to be extracellular could then be expressed as a percentage of total muscle water ('permeations' in Conway & Fitzgerald's (1942) terminology) and compared with the chloride and copper 'permeations' of the muscle, that is, the chloride and copper contents of muscle water expressed as percentages of those in the plasma water.

Before the injections some blood was withdrawn equal in volume to that of the solution to be injected. Inulin (Kerfut) was used in a 3% solution in sea water (2 ml. into animals of about 150 g.), sucrose and sodium thiosulphate in isosmotic solution (usually 1 and 0·5 ml., respectively). Inulin was determined photometrically in zinc hydroxide filtrates by the method of Roe, Epstein & Goldstein (1949), sucrose by the same method or by Somogyi's (1945) copper reagents after hydrolysis of the sucrose in the filtrates with an invertase preparation, and sodium thiosulphate in a tungstic acid filtrate according to Gilman, Philips & Koelle (1946). Choride was estimated in other aliquots of the same muscle filtrates by Sendroy's silver iodate method or the Volhard titration (Robertson, 1960*c*).

A comparison of the copper content of plasma and muscle enables an estimate to be made of the volume of blood in the muscle, since haemocyanin of the plasma is the chief copper-containing compound in the tissues. This comparison gives a maximum value for the blood-space in the muscle, since traces of copper inside the cells would reduce the estimate. While copper can be determined in ashed blood plasma or in trichloracetic extracts by direct colorimetric measurement of the copper-diethyldithiocarbamate complex, this fails with the muscle of *Nephrops* (and cephalopods) because the traces of zinc in the tissue precipitate and cause cloudiness of the solution. The complex was therefore extracted with amyl alcohol according to the method of Eden & Green (1940), but trichloracetic acid filtrates of blood and muscle were substituted for the preliminary ashing procedure of these authors.

In all muscle analyses involving the production of filtrates, measured quantities of reagents were added to weighed samples which were then ground thoroughly with quartz sand in a mortar, and aliquots of each filtrate were taken for analysis. Since the water content of the muscle was also determined, the results of analyses could be expressed in relation to water content.

Measurements of total osmotic concentration were made by a thermo-electric vapour-pressure method (Krogh, 1939, p. 211) on the plasma and muscle-juice of specimens, the juice being obtained with a small tissue-press (Krogh, 1938). By working rapidly the measurements on the juice could be completed within 20–60 min. of removing the muscle, thus minimizing changes due to breakdown of labile compounds.

Some inorganic analyses were made on the muscle-juice by the methods used for blood and whole muscle, but the small amount of magnesium was determined in the ash by means of titan yellow (Heagy, 1948).

II. INORGANIC IONS

(1) *Composition of whole muscle and plasma*

Three comparisons of muscle, plasma and sea water show close agreement (Table 1). Total concentrations of inorganic ions in the plasma and sea water are practically identical in two cases, within 1·8% of each other in the third. Ionic regulation in the plasma consists of increases in sodium and calcium over sea-water values, marked decreases in magnesium and sulphate, slight decreases in potassium, and virtual identity of chloride with that of the water; this is in agreement with a previous study (Robertson, 1949).

Table 1. *Ionic composition of whole muscle and blood plasma*

	mg. ions/kg. water								
	Na	K	Ca	Mg	Cl	SO_4	Total P	Total mg. ions	Water (g./kg. or g./l.)
(1) Muscle	84·4	173·5	5·24	20·6	120·7	4·2	149·8	558	749
Plasma	527	8·5	13·9	9·3	530	16·8	—	1106	947
Sea water	456	9·7	10·0	52·0	533	27·4	—	1088	986
(2) Muscle	83·2	159·2	5·26	16·9	91·3	2·1	141·5	500	752
Plasma	513	7·7	17·1	10·9	528	17·5	—	1094	935
Sea water	458	9·8	10·1	52·3	536	27·6	—	1094	986
(3) Muscle	82·1	167·0	5·12	19·8	117·8	3·0	143·0	538	768
Plasma	512	9·6	17·5	10·9	522	21·7	—	1094	946
Sea water	458	9·8	10·1	52·3	536	27·6	—	1094	986
Means									
Muscle	83·2	166·6	5·21	19·1	109·9	3·1	144·8	532	756
Plasma	517	8·6	16·2	10·4	527	18·7	—	1098	943
Sea water	457	9·8	10·1	52·2	535	27·5	—	1092	986

The figures for P (trichloracetic acid-soluble P) are really mg. atoms. The mg. ions are slightly lower, since the adenosine triphosphate molecule has 3 atoms P.

The sum of inorganic ions and acid-soluble phosphorus in muscle comes to only one-half of the ionic concentration of the plasma in each case, therefore the other half of the osmotic concentration (assuming identity between muscle and blood concentrations) must be made up chiefly by organic compounds other than those of phosphorus (see §III below). The pattern of ions in the muscle relative to the plasma is consistent: higher concentrations of potassium and magnesium, and lower concentrations of sodium, calcium, chloride and sulphate, with very high levels of acid-soluble phosphorus. Magnesium in the plasma is kept low partly by its selective excretion in the secretion of the antennal glands (Robertson, 1949); the approximate doubling of its concentration in the water of the muscle still leaves it well below the sea-water level.

(2) *Apparent extracellular spaces in muscle*

Tables 2 and 2*a* give the results of experiments with injected solutions of inulin, sucrose and sodium thiosulphate, with simultaneous measurements of chloride and copper. The samples of muscle taken for the analyses were quite large (*ca.* 8–10 g.)

in relation to the total abdominal flexor muscles, so that they should be fully representative and allow of replicate analyses. The chloride space is obtained by expressing the chloride concentration of the muscle as a percentage of the chloride of the plasma (both on a water-content basis). If there were no chloride inside the cells, the space would represent the volume of muscle water which is extracellular, having the same chloride concentration as the plasma.

Table 2. *Apparent extracellular spaces or 'permeations' in* Nephrops *muscle (concentrations in muscle as percentages of those in plasma, on water-content basis)*

	Extracellular spaces as % total muscle water			Muscle chloride mg./g. water	Time allowed for distribution (hr.)
	Chloride	Inulin	Copper		
	20·86	9·26	—	3·84	1
	19·87	11·02	4·85	3·61	2
	18·16	10·63	3·02	3·38	2
	18·22	16·22	—	3·33	3
	21·06	14·16	—	3·85	3
	22·06	12·41	4·94	4·06	6·5
	20·95	11·32	3·44	3·82	10
	19·81	10·72	3·39	3·53	10
	—	14·38	5·00	—	2*
	—	10·70	—	—	3*
Mean	20·12	12·08	4·11	3·68	—
S.E.	±0·49	±0·69	±0·37	±0·09	—

* Antennal gland openings closed with dental cement.

Table 2*a*. *Apparent extracellular spaces*

Extracellular spaces as % total muscle water				Muscle chloride mg./g. water	Time allowed for distribution (hr.)
Chloride	Sucrose	Sodium thiosulphate	Copper		
17·29	17·43	—	4·60	3·07	2
15·50	17·29	—	—	2·75	2
15·95	14·94	—	—	2·81	4
18·63	—	18·63	—	3·09	1

In *Nephrops* the chloride space is usually the largest, but it is equalled by one estimation of the sodium thiosulphate space, and exceeded by the sucrose space in two out of three cases. The copper space is the smallest. It represents the plasma water in the total muscle water, the copper being derived from the protein haemocyanin.

Inulin spaces are always lower than the corresponding chloride spaces, and two to three times as large as the copper spaces. They range between 9 and 16%, with a mean value of 12%. For the following reasons the inulin space has been taken as approximating the extracellular space:

(1) Theoretically, the large size of the inulin molecule, (M.W. about 5000) should make it difficult for this carbohydrate to enter cells.

(2) The inulin spaces are smaller than the sucrose and sodium thiosulphate spaces, and fairly consistent, after periods of 1–10 hr.

(3) The smaller molecule of sucrose (M.W. 342) and thiosulphate (ionic weight 112) give spaces practically equivalent to chloride spaces.

The simplest assumption is that the distribution of inulin gives a measure of extracellular fluid, and that the smaller sucrose molecule and thiosulphate ion have been able to diffuse to a small extent into the cells during the 1–4 hr. of the experiments. Sucrose is known to diffuse slowly into amphibian muscle cells (Krogh & Lindberg, 1944), while inulin seems to measure extracellular space in mammalian muscle (Conway & Fitzgerald, 1942).

On this basis, there is a small amount of chloride inside the muscle cells of *Nephrops* (see below, Table 3). This agrees with work on isolated muscle fibres of *Carcinus*, another marine decapod crustacean, in which there are about 53 mg. ions/kg. intracellular water (Shaw, 1955).

Accepting an inulin space of 12 % as the extracellular volume in total muscle water, and an average copper space of 4 % as the volume of plasma, the difference of 8 % would be a measure of interstitial fluid volume.

(3) *Intracellular ionic concentrations of muscle*

By subtracting from the mean concentrations of ions per kilogram muscle water (Table 1) values representing 120 ml. extracellular fluid (based on the composition of plasma), and then expressing the remaining intracellular concentrations in 880 ml. as concentrations per kilogram water, the intracellular composition is found (Table 3).

Table 3. *Intracellular composition of* Nephrops *muscle compared with blood plasma*

	mg. ions/kg. water									
	Na	K	Ca	Mg	Cl	SO_4	HCO_3	Lactate	P	Total
Muscle cells	24·5	188	3·72	20·3	53·1	1·02	1·90	8·8	164·2	466
Plasma	517	8·6	16·2	10·4	527	18·7	4·13	0·26	0·81	1103
Ratio: $\frac{\text{muscle cell}}{\text{plasma}}$	0·047	21·9	0·23	1·97	0·101	0·055	0·46	33·8	203	0·42

Water content of cells—731 g./kg.

The chief differences between intracellular and whole muscle concentrations are the reduced sodium and chloride values of the former, because of the subtraction of the sodium- and chloride-rich extracellular fluid. This results in a lower total concentration of analysed ions, 466 against 532 mg. ions. Potassium and magnesium are the only cations to become concentrated in the cells, the former to about twenty times and the latter to twice its level in the plasma. Sodium and sulphate fall to about a twentieth of their plasma values, calcium to a quarter, and chloride to a tenth.

If we assume that most of the cellular potassium is held electrostatically by non-diffusible organic anions, particularly organic phosphates, and that the low value for sodium is maintained by a process of active extrusion of sodium ions, and, further that the cells are permeable to small ions (e.g. Ussing, 1949; Hodgkin, 1951), it is possible that cellular potassium stands in Donnan equilibrium with the outside potassium, and

$$\frac{[K_i]}{[K_o]} = \frac{[Cl_o]}{[Cl_i]}.$$

In *Nephrops* these ratios are respectively 21·9 and 9·9. For these to represent a Donnan equilibrium, 50 % of the potassium would need to be in an unionized complex.

Evidence for the binding of about a quarter of the potassium is given in § IV, 2*b*. If this is accepted, the concentration of potassium above about 135 mg. ions or a 15-fold increase over the plasma value must be maintained by an active metabolic process.

III. ORGANIC CONSTITUENTS

(1) *The phosphorus compounds*

By separation of the barium-soluble and barium-insoluble compounds in an ice-cold trichloracetic acid extract of muscle, and by determining the total acid-soluble phosphorus of the extract, four main fractions were obtained: inorganic phosphorus, by direct estimation on a solution of the twice-precipitated barium salts; adenosine triphosphate (ATP) phosphorus from calculations on the increase in inorganic phosphorus after heating a similar volume of this solution with 1 N-HCl for 7 min. in a boiling water-bath, thus liberating two of the phosphate radicals of the compound; arginine phosphate phosphorus by taking a sample of the neutralized centrifugate and heating it in the presence of 2% trichloracetic acid for 1 minute in a boiling water-bath before estimating phosphorus; and a fourth fraction, chiefly hexose monophosphate, by subtracting the sum of the first three from the total acid-soluble phosphorus.

Table 4. *Acid-soluble phosphate fractions in* Nephrops *muscle*

	mg. ions/kg. water				
Specimen no.	Inorganic phosphate	Arginine phosphate	Adenosine triphosphate	Hexose phosphate, etc.	Total phosphate
1	30·0	51·8	—	—	—
2	35·3	57·2	—	—	—
3	22·3	67·6	13·4	10·5	113·8
4	18·1	65·2	13·0	8·5	104·8
5	14·4	80·1	8·3	24·2	127·0
Means 3–5	18·3	71·0	11·6	14·4	115·2*
S.E.	±2·3	±4·6	±1·6	±4·9	±6·4

* 115·2 mg. ions = 138·3 mg. atoms P (cf. 144·8 mg. atoms for specimens in Table 1).

The data are given in Table 4. Arginine phosphate is lower and inorganic phosphorus higher in the first two results, probably because of some breakdown of the phosphagen, since the last three results show greater consistency with practice of the technique. The labile arginine phosphate forms about 62% of the total phosphorus, inorganic phosphate 16%, ATP 10% and the remaining fraction 12%.

If all these constituents are free in the muscle cells, they would together amount to 10% of the osmotic concentration.

(2) *Nitrogenous constituents*

The chief non-protein nitrogeneous substances in *Nephrops* muscle have been found to be free amino acids, trimethylamine oxide, and glycine betaine; there is only a trace of volatile base.

By paper partition chromatography it is easy to identify the chief amino acids in a trichloracetic acid extract as glycine, taurine, arginine and proline, with lesser quantities of alanine, valine and aspartic and glutamic acids. These are similar to those found in an extensive study of the muscle of the lobster *Homarus* by Kermack *et al.* (1955), and by Camien, Sarlet, Duchâteau & Florkin (1951) in the muscles of *Homarus* and the crabs *Maia* and *Eriocheir*.

From the standpoint of the present investigation it was deemed sufficient to estimate total α-amino N in tungstic acid filtrates of *Nephrops* muscle by Folin's naphthoquinone sulphonate reagent as used by Russell (1944), although this photometric method may not be as accurate as manometric analysis, according to Chinard & Van Slyke (1947).

It was found that taurine N reacted with the naphthoquinone and was measured with the α-amino N of the other amino acids. Proline did not develop the full colour expected on the basis of its amino N, and, as found previously by Chinard & Van Slyke, is subject to fading. On the basis of a glycine standard (used throughout) it gave 70% of the theoretical colour 15–25 min. after final dilution. Some estimations of proline were therefore made using the method of Troll & Lindsley (1955), so that corrected figures could be given for total amino acids.

Table 5. *Nitrogenous constituents of* Nephrops *muscle*

	mM./kg. water			
Specimen no.	Amino acids (α-amino N)	Trimethylamine oxide	Betaine	Chloride
1	481	—	—	79·2
2	457	—	—	86·7
3	482	—	—	99·2
4	441	—	—	—
5	445	49·2	66·0	137·9
6	—	63·0	58·5	130·3
7	455	39·1	94·0	89·8
8	339	62·5	63·0	105·8
9	499	50·8	61·4	105·1
10	539	36·1	70·2	98·8
11	551	53·9	68·6	104·8
12	531	27·4	50·8	84·0
13	494	39·6	65·3	87·2
14	—	115·6	48·6	—
15	—	59·6	76·4	—
16	—	87·4	41·5	—
17	—	72·9	44·4	—
18	—	28·4	82·3	—
19	—	60·9	—	—
20	—	53·4	94·6	—
21	—	102·9	—	—
Mean	476	59·0	65·7	100·7
S.E.	16·4	±5·9	±4·2	±5·2

Volatile base (ammonium ions) of specimens 19, 20 and 21 was 3·0, 3·4 and 2·4 mg. ions/kg. water.

These figures include all the arginine. While it was possible by working rapidly to get a tungstic acid filtrate in which only about half of the labile arginine phosphate had broken down (giving 56–61 mM. free arginine as against the mean of 71 mM. arginine phosphate (Table 4) and 93 mM. free arginine when splitting was complete), the analyses were usually delayed 12–24 hr., when most or all of the arginine compound had split into its components.

From Table 5 it is seen that amino acids varied from 339 to 551 mM./kg. water, with a mean of 476 mM., thus constituting a very large proportion of the osmotic concentration of whole muscle, some 40–50% (see §IV).

The nitrogenous bases trimethylamine oxide and betaine are present in about equimolar concentration, with means of 59 and 66 mM., respectively. The former was more variable than the latter. Kermack *et al.* found 75 and 67 mM. respectively per kilogram fresh muscle in *Homarus*, approximately 100 and 90 mM. per kilogram water of the muscle. Shaw's (1958*b*) figure for trimethylamine oxide in the muscle fibres of *Carcinus*, 90 mM., is of a similar order. From the osmotic standpoint these two bases form about 13% of the total particle concentration in *Nephrops* (§IV).

Table 5*a*. *Some amino acids of* Nephrops *muscle*

	mM/kg. water			
	Total α-amino N	Proline	Arginine	Glycine, taurine, etc. (by difference)
	445	118	93	234
	539	93	100	346
	551	73	71	407
	531	106	97	328
	494	120	106	268
Mean	512	102	93	317
S.E.	±19·2	±8·7	±6·0	±30·3

In three other specimens, glutamine averaged 28·4 mM./kg. water (27·3, 24·9, 33·0), using the method of Kermack *et al.* (1955).

In Table 5*a* are a few analyses of proline and arginine in specimens 5 and 10–13 of Table 5. They show that the concentrations of these amino acids are very similar.

(3) *Soluble carbohydrates*

Carbohydrates contributing to the osmotic pressure of muscle and plasma are sugars and lactic acid. Boyland (1928) has given some data for the muscles of lobsters. Expressed in relation to water content and molality, lower carbohydrates (calculated as glucose) were 3·4 (1·5–5·9) mM./kg. water, lactic acid 9·1 (4·4–13·3) mM. in *Homarus vulgaris*; for *Palinurus vulgaris* these were 4·0 (0·7–5·9) mM. and 19·7 (11·8–26·7) mM., respectively.

A few analyses of reducing sugar and lactate in *Nephrops* muscle and plasma are given in Table 6. The mean values for plasma, 0·8 mM. and 0·3 mg. ions, are of the same order as those in intermoult *Carcinus* (Robertson, 1960*b*). Reducing sugar values in muscle are about ten times as high as in the plasma, being somewhat higher than in Boyland's analyses. The significance of the values of reducing sugar obtained on filtrates of muscle by ferricyanide (Boyland) or copper reagents (the present analysis) is somewhat questionable, as glucose will form only part of the reducing substances, another fraction being the hexose phosphates, of which some at least have a reducing value when unhydrolysed.

True resting values for lactate are difficult to obtain. The lower values of specimens 5 and 6 were obtained by quickly cutting out the muscle and dropping it into weighed beakers containing ice-cold trichloracetic acid, weighing, and then grinding with quartz sand and more acid in a cold mortar. Perhaps the mean of the three lowest figures, 8·4, or 7·8 mg. ions for two males, would be nearer the values in the living animal at rest.

Table 6. *Soluble carbohydrates in muscle and plasma*

	Reducing sugar				Lactate			
	Plasma		Muscle		Plasma		Muscle	
	mg./l.	mM./kg. water	g./kg.	mM./kg. water	mg./l.	mg. ions/kg. water	g./kg.	mg. ions/kg. water
1	134·6	0·79	1·494	11·0	17·0	0·20	1·523	22·8
2	140·2	0·83	1·084	8·0	30·0	0·36	0·564	8·5
3	168·3	0·99	0·641	4·7	40·0	0·48	2·396	35·9
4	120·6	0·71	1·167	8·7	7·0	0·08	1·471	22·0
5	—	—	—	—	14·3	0·17	0·473	7·1
6*	—	—	—	—	—	—	0·641	9·6
Mean	—	0·83	—	8·1	—	0·26	—	17·7
S.E.	—	±0·06	—	±1·3	—	±0·07	—	±4·5

* ♀ specimen.

Table 7. *Osmotic concentrations of plasma and muscle-juice (Krogh–Baldes method)*

Specimen	Sea water	Plasma	Muscle-juice*	Plasma as % sea water	Muscle-juice as % plasma
1	3·122	3·170	3.262 (1·5 hr.)	101·5	102·9
2	3·034	3·037	3·037 (0·4 hr.)	100·1	100·0
3	3·012	3·068	3·133 (0·3 hr.)	101·9	102·1
4	3·022	3·019	3·131 (0·4 hr).	99·9	103·7
5	2·997	3·009	2·994 (0·9 hr.)	100·4	99·5
6	2·997	3·030	3·186 (0·9 hr.)	101·1	105·1
7	3·011	3·022	2·989 (0·8 hr.)	100·4	98·9
8	3·011	2·982	3·055 (0·5 hr.)	99·0	102·4
9	3·009	2·976	3·079 (1·3 hr.)	98·9	103·5
Mean	3·024	3·035	3·096	100·4	102·0
S.E.	±0·0128	±0·0193	±0·0302	±0·344	±0·703

* Figures in brackets are times between removal of muscle and completion of estimation.

Concentrations are expressed in relation to % NaCl solution (g./100 g. water). On standard NaCl solutions, S.D. of method was 0·75 % ($N = 9$).

IV. OSMOLALITY AND CATION-ANION BALANCE

(1) *Direct measurements of osmolality*

These are given in Table 7. The plasma of nine specimens was isosmotic with sea water within 1·9%, the mean value being 100·4% that of the medium. This is the usual finding in marine decapod crustaceans (Robertson, 1960*a*, *b*). Compared with the plasma the juice expressed from the abdominal flexor muscles of the same specimens was more variable in concentration; in six cases it was slightly but definitely hyperosmotic by 2–5%. Delay in measuring the vapour pressure of the juice or delay in putting a sample of muscle in the press resulted in higher positive differences from the plasma, plus 5–8 %. These high values are apparently due to the breakdown of labile compounds such as arginine phosphate, with a consequent increase in osmotic concentration. The mean difference between plasma and muscle-juice was 2·0 % in the nine estimations done within 1·5 hr. of removing the muscle, 1·7 % in the seven done within an hour; the first difference is significant ($P < 0·05$), the second not, by the *t*-test.

It is reasonable to conclude that the mean difference of plus 2·0 % is due to partial breakdown of labile compounds during the working of the press at room temperature (17–19° C), and that the muscle cells are in life isosmotic with the interstitial fluid and plasma.

Table 8. *Osmotic concentration of plasma, whole muscle, and muscle cells*

	mg. ions or mM./kg. water		
Constituent	Plasma	Whole muscle	Muscle cells
Sodium	517	83·2	24·5
Potassium	8·6	166·6	188
Calcium	16·2	5·21	3·72
Magnesium	10·4	19·1	20·3
Ammonium	0·28	2·93	3·3
Chloride	527	109·9	53·1
Sulphate	18·7	3·1	1·02
Bicarbonate	4·13	2·16	1·90
Lactate	0·26	7·8	8·8
Inorganic phosphate	0·63	18·3	20·7
Arginine phosphate	—	71·0	80·7
Adenosine triphosphate	—	11·6	13·2
Remaining acid-soluble phosphate	0·18	14·4	16·4
Amino acids	3·77	405*	460*
Trimethylamine oxide	—	59·0	67·0
Betaine	—	65·7	74·6
Reducing sugar	0·83	—	—
Total	1108	1045	1037

* Excluding arginine of arginine phosphate.

(2) *Osmolality by summation of chemical constituents*

From the data given in §II and III, we can calculate the osmolality by summing the mg. ions and millimoles non-electrolytes, neglecting for the moment the osmotic coefficients of the salts and nitrogenous compounds, and the possibility that some of the 'ions' are bound. This is set out in Table 8, in which the figures for the muscle cells have been calculated on the basis that plasma and interstitial water form an extracellular volume of 12 % of total muscle water. The reducing sugar value of 8·1 mM. in whole muscle (Table 6) has been omitted in Table 8, since presumably it is largely included under 'remaining acid-soluble P', that is, hexose phosphates, etc., of 14·4 mM. Similarly, to avoid including arginine twice, 71 mM. (the value for arginine phosphate) has been subtracted from the mean of 476 mM. amino acids in whole muscle (Table 5).

The agreement between the total concentrations in cells and plasma is fairly good. However, the apparent balance is subject to several uncertainties, of which one is possible error involved in adding up constituents from different animals. Thus one series of specimens was used for inorganic ions, a second series for the phosphate compounds, and two further series for the nitrogenous compounds. This error was minimized by using animals in the spring and summer months, and in as healthy a condition as possible 3–4 days after being trawled.

Two further uncertainties are the possibilities that some of the ions are not free but bound to protein molecules, and that the osmotic coefficients of the salts (or ions) and organic compounds in solution may depart from unity.

(*a*) *Departure from ideal laws of solutions.* If the term 'milliosmoles' (often taken as equivalent to mg. ions in an electrolyte) is used as suggested by Dick (1959) as a unit of osmotic pressure, then a solution of Δ 1·858° C. contains 1000 m-osmoles. Sea water of this freezing-point depression contains 1129 mg. ions per kilogram solvent water, and is isosmotic with 0·553 M-NaCl (Sverdrup, Johnson & Fleming, 1942; Robinson, 1954).

It is probably safe to assume that the predominately inorganic plasma ions have proportionally the same osmotic concentration as the ions of sea water; thus the 1108 mg. ions would equal 981 m-osmoles. But the muscle cell contains a mixture of ions and organic compounds. In the cell solution one might assume that the amino acids and other nitrogenous compounds would behave approximately as ideal non-electrolytes, and that the inorganic ions and phosphates would behave like plasma ions.

At the molal concentration of 0·98, the osmotic concentrations of the amino acids and nitrogenous bases would be in proportion to their partial molal concentration, taking into account the osmotic coefficients. The latter do not depart from unity in the cases of several amino acids and betaine for which data are available. Thus at 1 molal the coefficients are 0·928 for glycine (Smith & Smith, 1937), 1·046 for proline and 1·115 for betaine (Smith & Smith, 1940), and 1·003 for alanine which behaves like an almost ideal solute (Robinson, 1952).

Assuming about 200 mM. glycine and 120 mM. proline, the 320 mM. of these two amino acids would equal 311 m-osmoles. The betaine concentration of 74·6 mM. equals 83·2 m-osmoles. Thus the sum of these three constituents, 395 mM., equals 394 m-osmoles, a rather fortuitous result. Lacking any information on the other amino acids and on trimethylamine oxide, we may assume for present purposes ideal behaviour, like alanine: the 602 mM. nitrogenous constituents would equal 602 m-osmoles.

Accepting the same average osmotic coefficient for cellular ions as for sea water and plasma (0·886), the 436 mg. ions would equal 386 m-osmoles, giving a total cellular osmolality of $602+386 = 988$ m-osmoles, a figure very close to the plasma value of 981.

(*b*) *The binding of ions.* When *Nephrops* muscle is subjected to pressure in a tissue-press an almost clear fluid is obtained which has a water content rather lower than that of the plasma, 914–936 g./kg., but much higher than that of whole muscle, 740–765 g./kg. In composition this muscle juice is quite different from the plasma, although isosmotic with it (Table 7), and differs in several respects from whole muscle, as shown in Table 9. On a water-content basis its chloride content is practically identical with that of muscle, but sodium and potassium are reduced by about a quarter, magnesium by over a half, calcium by nine-tenths and acid-soluble phosphorus by a fifth.

Table 9. *Composition of muscle-juice*

	mg. ions/kg. water						Water (g./kg.)
	Na	K	Ca	Mg	Cl	P*	
Muscle	87·6	165·3	7·91	23·7	94·8 (±2·9)	163·2	754 (±1·3)
Muscle-juice	66·5	122·8	0·79	9·46	94·6 (±2·4)	127·2	930 (±1·8)
Juice as % muscle	76	74	10	40	100	78	—
No. of paired estimations	2	2	1	1	6	1	7

* mg. atoms. (±) = S.E.
Four unpaired analyses for Mg were: muscle 11·5, 16·2, muscle-juice 4·04, 5·63 mg. ions/kg. water.

Table 10. *Approximate amount of ion-binding (based on data of* Table 9*)*

	mg. ions/kg. solvent water					
	Na	K	Ca	Mg	Cl	P*
Muscle cell	29·1	186·6	6·8	25·5	35·9	185·2
Cellular portion of muscle-juice †	5·1	138·3	0	9·3	35·7	144·4
Free ions	18 %	74 %	0 %	36 %	99 %	78 %
Bound ions	82 %	26 %	100 %	64 %	1 %	22 %

* mg. atoms.
† I.e. excluding extracellular component of juice.

It seems impossible to escape the conclusion that these reductions are due to the holding back by proteins of some of the 'ions', those bound in complexes. Neither contractile proteins nor the soluble proteins of the sarcoplasm appear to cross the cell walls under pressure. Only the free ions, including all the chloride of the cell, pass out in the expressed juice, together with the amino acids and nitrogenous bases. Collectively, the osmotic concentration of all these ions and nitrogenous compounds is 980–1000 m-osmoles, making the muscle-juice isosmotic with the plasma (see Table 7). It is these free constituents that make up the osmotically active portion of the muscle cell.

The proportion of extracellular fluid in the muscle-juice has not been measured, but is presumably the same as that in whole muscle, about 12% of the water content. Accepting this, and assuming that the average composition of the plasma (Table 8) represents that of extracellular fluid as a whole, appropriate calculations can be made for the composition of the muscle cells and cellular portion of the muscle-juice in these specimens. The calculated composition is given in Table 10.

All the calcium of the cell seems to be bound, as well as over 60% of the magnesium, 82% of the sodium and 26% of the potassium, while none of the chloride is bound and only 22% of the phosphorus. Which phosphorus compounds are bound have not been determined, possibly ATP (Dubuisson, 1942), as this would reduce the number of phosphorus atoms by 25%.

If these corrections are applied to the ions of the muscle cells as given in Table 8, the 436 mg. ions become 337 mg. ions, which when multiplied by the average osmotic coefficient of 0·886 are equivalent to 299 m-osmoles. A revised osmotic balance sheet can now be made up (Table 11). The correction for ion-binding in the plasma is very small, as it concerns only 12% of the calcium (Robertson, 1949). About 92% of the osmotic concentration in the cell has been accounted for by free ions, amino acids and nitrogenous bases.

Table 11. *Osmotic concentration of plasma and muscle cells*

	mg. ions or mM./kg. water		m-osmoles/kg. water: Uncorrected		m-osmoles/kg. water: Corrected for ion binding	
	Plasma	Muscle cells	Plasma	Muscle cells	Plasma	Muscle cells
Inorganic ions and phosphates, etc.	1104·2	435·6	977	386	976	299
Amino acids	3·8	460	4	460	4	460
Nitrogenous bases	—	142	—	142	—	142
Total	1108	1037	981	988	980	901

(3) *Cation-anion balance*

This may be calculated from the data of Table 8 by taking valency into account. As far as most of the amino acids are concerned, their positive and negative charges are assumed to balance out, except in the case of arginine with its two amino groups. The difference between total arginine and arginine phosphate in whole muscle, with means of 93 and 71 mM./kg. water, respectively, suggests that about 22 mM. are free. This equals 25 mM. intracellular arginine.

Since the valency of the phosphate compounds varies with pH, an attempt was made to estimate intracellular pH by making glass electrode measurements on blood, ground muscle, muscle-mince, muscle-press juice and a centrifugate of muscle. A little distilled water was added during the grinding of the muscle in a mortar, and to the small amount of clear centrifugate; the fluids thus obtained were well buffered. The results are given in Table 12. Calculation of the intracellular pH has been made assuming that the 12% extracellular fluid has the pH of the blood.

Table 12. *Measurements of* pH (*glass electrode*)

	Blood	Ground muscle	Muscle-mince	Muscle-juice	Muscle centrifugate
	7·66	6·97	—	—	—
	7·70	6·94	—	6·60	—
	7·84	6·95	6·98	6·96	6·82
		6·96*	—	6·59*	—
Calculated intracellular pH		6·91	6·93	6·53–6·91	6·77

* Second sample.

It is of course impossible to be certain that the pH inside an intact muscle cell approaches that found in ground tissues, even when the extracellular component has been allowed for. Stimulation by grinding and pressing may cause breakdown of arginine phosphate which would have an alkalinizing effect, and the production of lactic acid with an acidifying effect. At their face values, however, the results suggest an internal pH of 6·8–6·9 in the muscle cells. These values are similar to those of Caldwell (1958) on *Carcinus* muscle.

In Table 13 the equivalents of the phosphate compounds have been calculated for both pH 6 and 7.

Table 13. *Cation-anion balance*

	m.equiv./kg. water		
		Muscle cell constituents	
Ion	Plasma	Free + bound	Free
Sodium	517	24·5	4·4
Potassium	8·6	188	139
Calcium	32·4	7·4	0
Magnesium	20·8	40·6	14·6
Ammonium	0·3	3·3	3·3
Arginine (net $+^{\text{ve}}$ charge)	—	25	25
Total cations	579·1	288·8	186·3
Chloride	527	53·1	53·1
Sulphate	37·4	2·0	2·0
Bicarbonate	4·1	1·9	1·9
Lactate	0·3	8·8	8·8
Inorganic phosphate	1·2	33·0*–23·1	33·0*–23·1
Arginine phosphate	*ca.* 0·4	83·9–80·7	83·9–80·7
Adenosine triphosphate		50·1–42·9	—
Hexose monophosphate, etc.		31·8–23·6	31·8–23·6
Total anions	570·4	264·6–236·1	214·5–193·2

* Assuming intracellular pH of 7·0; second figure for pH 6·0. Equivalents of phosphates calculated from data of Sörensen (1912): inorganic phosphate; Meyerhof & Lohmann (1928): arginine phosphate; Alberty, Smith & Bock (1951): adenosine triphosphate; Meyerhof & Lohmann (1927): hexose monophosphate.

The small deficiency of anions in the plasma is probably made up by proteinate. In the muscle cells about three-quarters of the anions investigated are phosphates, and the deficiency of 24–53 m-equiv. in the third column may be met by proteins. This has not been investigated in *Nephrops*. Dubuisson (1954) calculates that myosinate and myogenate in frog muscle cells at pH 6·8 may contribute 24 (22 + 2) m-equiv. to the anions, but doubts whether they are free. Conway's (1957) estimates of the charges of myosin and myogen of rat muscle are much greater, 61 m-equiv. at pH 7 and 30 at pH 6.

However, it is certain that some of the other constituents are bound wholly or in part in complexes, and in the last column of the table are given the estimates of free ions, based for inorganic ions on the composition of the cellular portion of muscle-juice. Presumably the sarcolemma, myosin and actin are the chief binders of the ions, removing all the cellular calcium and most of the magnesium and sodium from free solution, as well as a small proportion of the potassium. It has been assumed also that the adenosine triphosphate is bound to myosin and that the only proteins to be considered are those of the sarcoplasm, the myogen complex, whose net contribution to free anions may be quite small.

Cation-anion balance is fair, considering the uncertainties and difficulties of interpretation, and would be satisfactory if the intracellular pH were closer to 6 than to 7.

V. ALTERATIONS IN THE BLOOD-MUSCLE STEADY STATE

The ionic steady state in the plasma of *Nephrops* and other decapod crustaceans, in which levels of ions are maintained different from those of the surrounding sea water despite some degree of ionic permeability of the gills, is a process controlled by the antennal glands and gills with the expenditure of energy (Robertson, 1949; 1960*a*). Likewise, ionic and amino acid regulation in muscle, in which levels are maintained often vastly different from those of the plasma, requires energy. Death of a lobster brings about changes in the permeability of the cells, with a tendency towards equalizing the concentrations of ions and nitrogenous compounds in plasma and muscle.

Table 14. *Changes in the plasma-muscle steady state after death*

	mg. ions or mM./kg. water			
	Plasma		Muscle	
	Cl	α-amino N	Cl	α-amino N
Normal*	527	3·8	110	476
Dead 12 hr.	516	2·3	105	475
Dead 24 hr.	364	161·6	278	316

* Mean data from Tables 1, 5 and 8.
The dead animals were kept at 14–16° C.

In Table 14 are given chloride and α-amino N analyses of plasma and muscle of two dead specimens, one 12 hr., the other 24 hr. after respiratory movements and the antennal reflexes had ceased. The first specimen showed no differences from normal animals, but the second showed marked changes; chloride had fallen to about 70% in the plasma and increased to nearly three times its usual value in the muscle, whereas amino N had fallen in the muscle and increased in the plasma from the order of 2–4 mM. to 162 mM. These changes imply leakage of amino acids from muscle cells to extracellular fluid, and permeation of the muscle cells with chloride from the plasma, both processes tending to equalize the concentrations on both sides of the cell membranes.

Since the gills or integument of most marine invertebrates including crustaceans are permeable to the ions of sea water (Krogh, 1939), an attempt was made to ascertain whether changes brought about in the plasma by altering the levels of ions in the sea water would cause corresponding changes in the muscle.

Two *Nephrops* were kept for 3 days in sea water in which the potassium and calcium concentrations had been increased to about three times normal by adding approximately isosmotic solutions of the chlorides. Inspection of Table 15 shows that potassium of the plasma was much higher than in normal animals, having increased in proportion to the difference in external level; muscle potassium was higher by only 10 and 18%. Calcium of the plasma was up to twice, and in the muscle to three and five times, the usual concentration. *T*-tests showed that the differences in the potassium and calcium levels of the muscle were significant.

To be certain that the intracellular concentrations increased would require knowledge of the extracellular phase in the muscle of these specimens. This can be assumed to be of the same order as found previously in normal sea water (§II, 2). On this basis the intracellular concentrations in mg. ions/kg. water are: K 188 ± 4·62 (normal) and 212·9 ± 5·96 (K- and Ca-rich); Ca 3·71 ± 0·19 and 17·43 ± 4·94; these differences are significant in each case ($P < 0{\cdot}05$). It is therefore probable that the increased concentrations in muscle are in both the extracellular and intracellular phases.

The experiment leads to the conclusion that the level of potassium and calcium ions in plasma and muscle in *Nephrops* is broadly dependent on the concentrations of these ions in sea water.

Table 15. *Changes in ions of plasma and muscle in sea water with raised levels of potassium and calcium*

	mg. ions/kg. water			mg. ions/kg. water		
	Sea water	Plasma	Muscle	Sea water	Plasma	Muscle
Na	457	517	83·2	409	444	87·8
					461	60·0
K	9·8	8·6	166·6	27·1	25·1	183·7
					21·8	196·8
Ca	10·1	16·2	5·2	27·7	31·7	25·3
					36·2	13·5
Mg	52·2	10·4	19·1	46·7	15·7	19·5
					13·5	11·8
Cl	535	527	110	534	524	142
					524	101
SO_4	27·5	18·7	3·1	24·7	14·5	—
					17·8	—
Total	1092	1098	387	1069	1055	458
					1074	383
Water (mg./ml.) or (mg./g.)	986	943	756	986	945	762
					933	760
	Normal sea water, 3 specimens			K- and Ca-rich sea water, 2 specimens		

Data in normal sea water from Table 1.
t-test on muscle: K 166·6 ± 4·13 (normal) and 190·3 ± 6·55; Ca 5·21 ± 0·04 and 19·4 ± 5·9. Differences significant at $P = 0{\cdot}05$.

VI. DISCUSSION

It has long been known that the ash content of marine invertebrate muscle is about half that of the blood (Fredericq, 1904) and that the osmotic concentration must be made up by organic substances. The pattern of inorganic ions in crustacean and molluscan muscle has been studied by Bialaszewicz & Kupfer (1936) and Hayes & Pelluet (1947), and more recently by Potts (1958) and Shaw (1955): on a molar basis potassium usually exceeds sodium by a factor approaching two, calcium and magnesium are present in relatively low concentration, and chloride usually exceeds sodium. Only in the last 10 years has the quantitative importance of free amino acids in muscle been realized, by finding some 200–350 mM./kg. fresh tissue in lobsters (*Homarus*, *Nephrops*), crabs (*Carcinus*, *Eriocheir*) and mussels (*Mytilus*) (Camien *et al.* 1951; Kermack *et al.* 1955; Robertson, 1957; Shaw, 1958*b*; Potts, 1958). Of lesser importance, at least in crustaceans, are the nitrogenous bases trimethylamine oxide and glycine betaine which are present in concentrations of about 70 mM./kg. in *Homarus* muscle (Kermack *et al.*).

In the present paper analyses of whole muscle of *Nephrops* have been given for all these constituents and others including the principal organic phosphorus compounds. On a water-content basis the sum of ions and molecules comes to 94% of that in the plasma, the plasma itself being isosmotic with the external sea water within 1%. The great importance of non-protein amino acids of muscle is evident from the fact that on the average 405 mM. are present, 476 mM. if arginine phosphate is included. Quantitatively more important than arginine phosphate in the muscle are potassium, chloride and sodium ions, with trimethylamine oxide and betaine only slightly less concentrated than in *Homarus*.

Whole muscle includes an extracellular phase which seems to be measured by the inulin space or permeation, forming about 12% of the total muscle water. This inulin permeation was consistently less than that calculated from injections of sucrose or sodium thiosulphate; these latter substances appear to enter the cells slightly. From copper analyses a maximum blood space of 4% of the extracellular water has been estimated, and the difference of 8% has been attributed to the interstitial fluid round the individual cells of the muscle.

Zuckerkandl (1960) who has discussed various inconsistencies and anomalies in the measurement of blood volume in crustaceans is prepared to believe in the existence of a non-circulatory component of extracellular fluid from the fact that the dilution of the circulating blood at moult does not correspond with the volume of extracellular fluid (sea water) incorporated. It has been shown, however, that about a third of the water absorbed at moult by *Carcinus* is taken up by the cells; it does not all remain in the blood and interstitial fluid compartments (Robertson, 1960*b*). The finding of a space in muscle into which inulin and other substances diffuse and from which blood with its haemocyanin is excluded gives experimental support for an interstitial phase, but the morphological separation of blood and interstitial fluid has still to be worked out.

Intracellular concentrations of ions and molecules in *Nephrops* muscle obtained by appropriate corrections for the 12% extracellular fluid do not differ in total by more than 1% from those of whole muscle. Concentrations of sodium and chloride change from 83 and 110 mg. ions to 25 and 53 mg. ions, respectively, because of the high content of these ions in the extracellular fluid. Most of the other inorganic ions and all the organic compounds show a higher level inside the cells than in whole muscle, for example, potassium 188 compared to 167 mg. ions, and the amino acids 460 compared to 405 mM./kg. water.

The only data comparable in scope are those of Shaw (1955, 1958*a*, *b*) on muscle fibres of the chela of *Carcinus maenas* prepared by a method involving washing with a solution of isosmotic glucose to remove the extracellular fluid. Despite the two different methods of obtaining intracellular concentrations, the pattern of results on the two animals is remarkably similar. In mg. ions *Carcinus* has rather lower figures for potassium (146) and arginine phosphate (59), a slightly higher sodium (54), and an identical chloride concentration (53).

Shaw (1955) was inclined to accept the concept of a Donnan equilibrium between external and internal concentrations of potassium and chloride for *Carcinus* in normal strength sea water, finding on average

$$\frac{[\mathrm{K}_i]}{[\mathrm{K}_o]} = 9{\cdot}4 \quad \text{and} \quad \frac{[\mathrm{Cl}_o]}{[\mathrm{Cl}_i]} = 10{\cdot}3.$$

Later findings (Shaw, 1958*a*) of a higher mean potassium ratio of 12 (11·2–13·6) are against this, unless some 20% of the intracellular potassium is bound in a protein complex. In *Nephrops* the corresponding ratios are quite unequal, 21·9 and 9·9.

Alterations in the external medium can cause alterations in the ions of muscle through changes in the blood. Thus the potassium contents of *Carcinus* muscle can be lowered by keeping crabs in an artificial sea water of reduced potassium content (Shaw, 1958*a*). Similarly, the potassium and calcium concentrations of *Nephrops* muscle can be raised by keeping this lobster in water of high potassium and calcium content.

Comparable analyses of other marine invertebrate muscles are few. *Mytilus* adductor muscles studied by Potts (1958) show similar intracellular concentrations of potassium and higher concentrations of sodium and chloride. The fast adductor muscle of *Pecten* is more like the crustacean muscles in these three ions. Unlike *Nephrops* and *Carcinus*, these bivalves do not reduce the magnesium content of the blood relative to that of sea water (Robertson, 1949), and this is correlated with higher intracellular concentrations of magnesium, at least in *Mytilus* where the fast adductor has a value of 34 mM./kg. fibre water, almost double that of the crustaceans.

Direct measurements of osmolality on juice pressed from *Nephrops* muscle have shown that the concentration of osmotically active substances increases with time, but if the measurements are made within 1 hr., the vapour pressure of the juice does not differ significantly from that of the plasma (mean difference +1·7%).

Cryoscopic methods applied to frozen ground mammalian muscle mixed with saline show a rapid increase in osmotic concentration in a few minutes (Conway & McCormack, 1953), due to the breakdown of ATP, hexose esters, creatine phosphate and glycogen (Conway, Geoghegan & McCormack, 1955), but extrapolation to zero time shows that the muscle and plasma are normally in osmotic equilibrium. This conclusion can probably be extended to other mammalian tissues, despite the contradictions in the literature (Robinson, 1960).

Among invertebrates, Potts (1952) found the freezing point of *Mytilus* muscle fibres to be within 1·5% of that of the blood, confirming Krogh's (1939) vapour-pressure determinations of muscle juice from this bivalve. But Shaw (1958*b*) found the muscle fibres of *Carcinus* to be 7–8% hyperosmotic to the plasma, using the same micro-freezing point method used by Potts. Shaw stored the fibres in glass capillaries at −80° C for an unstated period, and he attributed the result to the breakdown of arginine phosphate and ATP during storage. It is perhaps unfortunate that he did not use the full potentiality of the method by making some measurements immediately, before serious breakdown of labile compounds had taken place.

Analysis of the juice pressed from muscle in *Nephrops* shows it to contain little if any protein. In this respect it is unlike the juice obtained after grinding muscle in saline, from which the myogen fraction of muscle proteins is obtained. Apart from its extracellular fluid component, it should be an ultra-filtrate of the cells. If the proteins of the cells and all the ions were completely free in solution one would expect a Donnan equilibrium ratio in such ions as Na^+, K^+ and Cl^-, but the results (Table 10) were far from this expectation: Cl^- ions seemed to pass in equal concentration into the juice, but all the other ions including phosphates were kept back by cell constituents in variable degree, calcium completely.

A working hypothesis to explain the findings is to assume that most of the protein is not freely in solution, and thus does not impose a larger Donnan value for the chloride concentration of the juice. It is possible, alternatively, that a small fraction of the intracellular chloride is bound to proteins as Scatchard, Scheinberg & Armstrong (1950) find in sodium chloride solutions with serum-albumin, thus causing the Donnan ratio Cl_o/Cl_i to exceed 1. But the large apparent diminution in cellular Na^+, Ca^{++} and Mg^{++} to 18, 0 and 36%, respectively, in the juice, and the smaller diminution of K^+ and acid-soluble phosphate to 74% and 78% suggest that the variable decreases reflect the binding by cellular proteins and the sarcolemma of significant amounts of these 'ions' in osmotically inactive complexes.

Support for the binding of a proportion of the alkali-metal ions is still small (Robinson, 1960). Steinbach (1950) investigated a centrifuged homogenate of frog thigh muscles and found no potassium binding in the residue compared to the supernatant liquid, but a small amount of sodium binding (2–3% of the total). On the other hand, Stone & Shapiro (1948) found that only about 78% of the potassium in a homogenate of rat gastrocnemius muscle could diffuse across a collodion membrane. The older work of Quagliariello (1929) showed that 34% of the potassium, 44% of the calcium and 57% of the magnesium in the juice pressed from minced and ground dog muscle did not appear in an ultrafiltrate, presumably on account of binding by proteins in the juice.

Binding of much of the calcium and magnesium of muscle is less disputable. Apart from Quagliariello's results, Conway (1957) states from unpublished work that one-half of the calcium and magnesium of frog muscle fibres is probably bound to protein. Hasselbach (1957) has analysed different components of the structural proteins of rabbit muscle and finds much of the calcium and some of the magnesium bound to actin and L-myosin.

In drawing up a final osmotic balance sheet for *Nephrops* muscle cells allowance has been made for the fact that the 602 mM. of amino acids and nitrogenous bases are probably behaving as ideal non-electrolytes, while the 436 mg. ions of the cell, both inorganic ions and organic phosphates, have an over-all osmotic coefficient of about 0·89, which is that of a sea water having the same osmotic concentration as the cell. The calculated total in milliosmoles is then within 1% of that of the plasma, but if allowance is made for the probable binding of some of the four principal cations and adenosine triphosphate the final figure is 92% of the plasma value.

VII. SUMMARY

1. Comprehensive analyses have been made of the muscle and plasma for inorganic ions, organic phosphates, soluble carbohydrates, amino acids and nitrogenous bases.

2. Compared with a total of 1108 mg. ions/kg. water in the plasma, the muscle had 1045, of which 515 were mg. ions (Na, K, Ca, Mg, Cl, SO_4, lactate, HCO_3, inorganic and arginine phosphates, adenosine triphosphate, hexose phosphates, etc.), and the remainder amino acids (405 mM.) and the nitrogenous compounds trimethylamine oxide (59 mM.) and betaine (66 mM.).

3. Measurements of the apparent extracellular spaces in the muscle following injections of inulin, sucrose and sodium thiosulphate have led to the conclusion that

the inulin space or permeation, 12·1% ± 0·69 (S.E.) of the total muscle water, is the best indication of total extracellular fluid in the muscle. The copper space, 4·11 ± 0·37, represents a maximum value for the blood space (that filled with haemocyanin), leaving 8·0% as the volume occupied by interstitial fluid.

4. Intracellular concentrations of potassium and chloride do not conform to values expected on the basis of a Donnan equilibrium with plasma: $[K_i]/[K_o] = 21{\cdot}9$, $[Cl_o]/[Cl_i] = 9{\cdot}9$.

5. Mean values in mg. ions/kg. water for the whole muscle concentrations of phosphate ions are inorganic phosphate 18·3, arginine phosphate 71·0, adenosine triphosphate 11·6, remaining acid-soluble phosphate, chiefly hexose phosphates, 14·4, total 115·2.

6. Total non-protein amino acids of muscle from measurements of α-amino N are 339–551 mM., mean 476. These are chiefly glycine, proline, arginine, taurine, alanine and glutamine. Most of the arginine is combined as arginine phosphate.

7. Direct measurements of osmolality of the plasma using Krogh–Baldes thermocouples give a mean value of 100·4 ± 0·34 (N = 9), taking sea water as 100. Measurements of the juice expressed from muscle with a tissue-press give a mean value compared with plasma (100) of 102·0 ± 0·70 if done within 1·5 hr. of removing the muscle, 101·7 ± 0·87 for seven estimations done within an hour; the latter result is not significantly different from the plasma values. Delay in measurement leads to higher values (plus 5–8% on plasma), owing to the breakdown of labile compounds.

8. Compared with whole muscle on a water-content basis, the concentrations of ions in the muscle-juice are Na 76%, K 74%, Ca 10%, Mg 40%, Cl 100%, total acid-soluble P 78%. This is interpreted as showing binding by proteins of fractions of all these ions except chloride. If the juice is corrected for its extracellular component, approximate percentages of ions bound inside the muscle cells are Na 82%, K 26%, Ca 100%, Mg 64% and P 22%; all the cellular chloride is free.

9. A final osmotic balance sheet for *Nephrops* muscle cells, in which consideration has been given to such factors as binding of ions, osmotic coefficients of ions and probable ideal behaviour of the nitrogenous compounds, shows that about 92% of the total osmotic concentration (in milliosmoles) has been accounted for.

10. Increasing the concentration of potassium and calcium in sea water leads to increases in the values of these ions in both plasma and muscle.

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THE EFFLUX OF SODIUM IONS FROM THE LAST ABDOMINAL GANGLION OF THE COCKROACH, *PERIPLANETA AMERICANA* L.

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INTRODUCTION

The exchanges of sodium and potassium ions and of sugar molecules between the haemolymph and the central nervous system of the cockroach have been found to occur relatively rapidly (Treherne, 1960, 1961 *a*). A recent investigation has also shown that the measured efflux of sodium ions from the whole abdominal nerve cord of the cockroach was substantially reduced by the presence of metabolic inhibitors and the absence of potassium ions in the external solution (Treherne, 1961 *b*). These results clearly suggested that the rate-limiting transfer measured in these experiments was, in fact, some sort of linked-sodium pump. In the present experiments on the rate of loss of ^{24}Na from the isolated terminal abdominal ganglion an attempt has been made to extend the earlier observations and, in particular, to investigate the role of the cellular and fibrous nerve sheath, the perilemma, in the extrusion of sodium ions from the central nervous system of this insect.

METHODS

In these experiments the efflux of ^{24}Na from the isolated terminal abdominal ganglion was studied while maintaining the preparation in a stream of oxygenated physiological solution. The ganglion was made radioactive either by the injection of 100 μl. of a solution containing ^{24}Na (0·1–0·5 mc./ml.) into the haemolymph or by the perfusion of the whole abdominal nerve cord for 30 min. with the solution. The perfusion was carried out on decapitated adult male *Periplaneta americana* L. in which the dorsal integument was cut away and the viscera removed to expose the abdominal nerve cord. The tracheal supply of the nerve cord thus remained intact during the period when the body cavity was flooded with the radioactive solution. The whole radioactive nerve cord was then removed and the terminal ganglion isolated by ligatures at the anterior connectives and the cercal nerves. The ligatures were tied with threads pulled from 15 denier nylon stockings. The isolated ganglion was then tied to a small length of glass rod and mounted in the apparatus used to measure sodium efflux. This apparatus, which was used in an earlier investigation (Treherne, 1961 *b*), consisted of a small rectangular Perspex chamber (0·45 ml. volume) the floor of which was formed by 0·00025 in. Terylene sheet of minimal stopping power to the radiations emitted by ^{24}Na. Oxygenated physiological saline flowed through the chamber at a rate of approximately 0·85 ml./sec. A Geiger tube (Mullard MX. 123) was mounted beneath the Perspex chamber and continuous record was kept of the decline in radioactivity associated with the ganglion using a Panax scaler unit (100 c.).

In some experiments the isolated radioactive ganglia were partially desheathed according to the procedure outlined by Twarog & Roeder (1956). The sheath was lifted and torn gently from the entire dorsal surface of the ganglion using finely ground watchmaker's forceps. According to Twarog & Roeder desheathing involves removal of the outer connective tissue layer and the associated cellular perineurium.

The various solutions used in these experiments were similar to those devised for some previous investigations. The radioactive solution used for injection into the haemolymph and for perfusion of the nerve cord was that given by Treherne (1961 *a*). The physiological solutions used in the efflux experiments were the same as those devised for the investigation on the whole abdominal nerve cord (Treherne, 1961 *b*). These consisted of a normal solution containing the various ions and molecules in proportions approximately similar to those in cockroach haemolymph together with a modified potassium-free solution in which the KCl (12·3 mM./l.) was replaced by an appropriate increase in NaCl (to 167·1 mM/l.).

The sodium concentrations of the terminal abdominal ganglion were measured by means of an EEL flame photometer. For this purpose batches of three ganglia were weighed on a 5·0 mg. torsion balance and ashed on small pieces of platinum foil in a muffle furnace at temperatures of 460–480° C.

RESULTS

The measurements of the sodium content of the terminal abdominal ganglion are tabulated in Table 1. The mean value of 110·5 ± 19·8 mM./l. is of the same order as the sodium concentrations for whole nerve cords given by Tobias (1948) and Treherne (1961 *a*).

Table 1. *The sodium concentration* (mM/l. *tissue water*) *of the last abdominal ganglion*

Serial	Na content (mM./l.)	Mean
1	137·0	110·5 mM./l.
2	114·0	
3	126·1	
4	109·6	
5	107·8	
6	124·4	
7	85·1	
8	112·9	
9	78·8	

Fig. 1 shows the rate of disappearance of the radioactive sodium from an isolated terminal abdominal ganglion when washed in flowing inactive solution. The decline in radioactivity associated with the ganglion appeared to follow an approximately exponential fall for as long as the experiment could be continued. There was no evidence in this preparation of a final slow phase of sodium efflux in a region of low radioactivity such as was obtained with the whole nerve cord (Treherne, 1961 *b*). The average half time ($t_{0·5}$) for sodium efflux from the ganglion was 5·57 ± 1·01 min.

In the next group of experiments the effect of removing the connective tissue and cellular sheath from the dorsal surface of the ganglion was tested. In these experiments the initial exponential decline in radioactivity was determined. Following this the ganglion was removed from the apparatus and quickly desheathed. The partially desheathed preparation was then returned to the apparatus and the subsequent rate of sodium efflux determined. Fig. 2 shows a typical result obtained with this procedure. Removal of the sheath appeared to have little effect on the rate of loss of sodium ions from the terminal ganglion. The effect of partial desheathing of the ganglion on sodium efflux is summarized in Table 2.

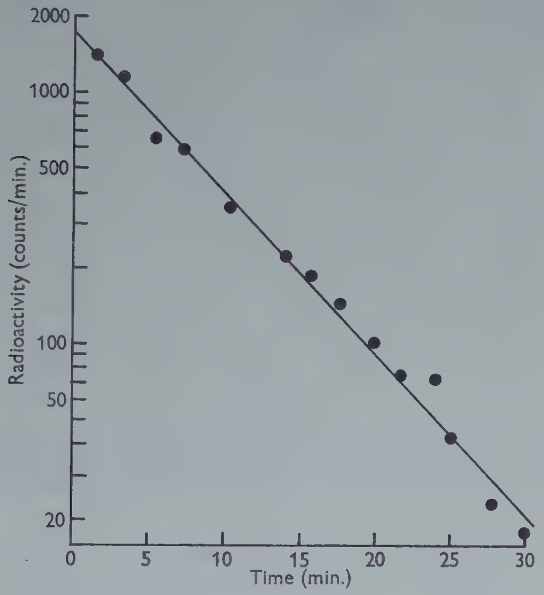

Fig. 1. A semi-logarithmic plot of the decline in radioactivity of a ligatured isolated abdominal ganglion when washed in inactive solution. The ganglion was made radioactive by perfusing the whole abdominal nerve cord with a solution containing ^{24}Na.

The effect of 0·2 mM./l. 2:4-dinitrophenol on the rate of loss of sodium ions from this preparation is illustrated in Fig. 3. Replacement of the normal solution with one containing the poison resulted in a clear-cut slowing down of sodium efflux. Return to the normal solution produced a rapid loss of radioactivity which was followed by an efflux that was slightly slower than the initial rate of sodium loss (Fig. 4). The effect of the presence of 2:4-dinitrophenol on the rate of sodium loss was also tested in the desheathed preparation. In Table 3 the effect of a 0·2 mM./l. solution of the poison on desheathed preparation is compared with that of normal ganglia. These results appeared to show that the poison had less effect on the desheathed as compared with the intact preparation ($P < 0·05$).

The effect of transferring the isolated ganglia to a potassium-free solution is illustrated in Fig. 5. In this case the extrusion of ^{24}Na clearly slowed down in the potassium-free solution. On return to the normal solution, containing 12·3 mM./l. K, there appeared to be a transient rapid efflux which was followed by a rate of sodium loss similar to the initial one. A comparison of the effect of potassium-free solution on normal and desheathed ganglia apparently showed that the effect was less pronounced in the desheathed preparations ($P < 0{\cdot}05$, Table 4).

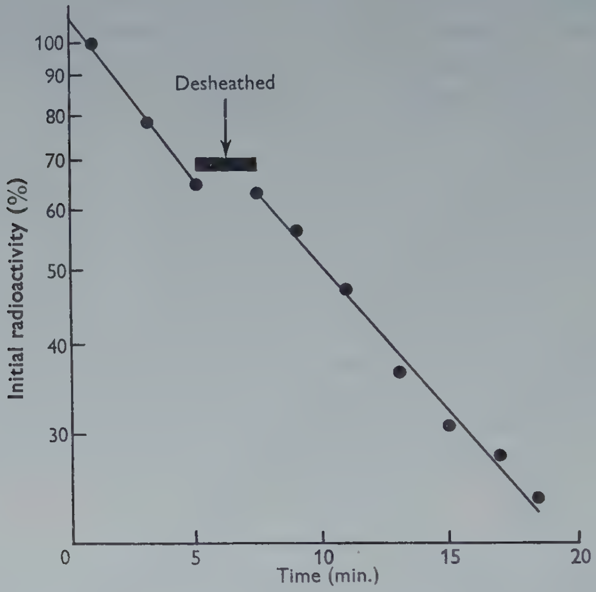

Fig. 2. The effect of removing the connective tissue and cellular sheath from the dorsal surface of the ganglion on the rate of loss of ^{24}Na when washed in inactive solution.

Table 2. *The effect of removal of the perilemma on the half time of sodium loss from the last abdominal ganglion*

Serial	Initial $t_{0{\cdot}5}$ (min.)	Desheathed ganglion $t_{0{\cdot}5}$ (min.)
1	5·0	5·3
2	4·7	4·8
3	5·6	5·1
4	6·0	5·9
5	6·8	7·8

DISCUSSION

It is of some interest to compare these results for the isolated abdominal ganglion with those obtained from experiments on the efflux of sodium ions from the whole nerve cord and from isolated connectives (Treherne, 1961*b*). The sodium content of the terminal ganglion was of approximately the same level as that of the whole abdominal nerve cord (Treherne, 1961*a*) so that the rates of efflux, as measured by the decline in radioactivity, are more or less comparable in the different preparations. The values for $t_{0{\cdot}5}$ appeared to be approximately similar for the connectives (Treherne, 1961*b*) and for the terminal abdominal ganglion used in the present experiments.

These results are perhaps rather unexpected in view of the obvious difference in shape between these two structures. Measurements showed that the surface/volume ratio of the connectives was about 3·5 times greater than that of the relatively massive terminal abdominal ganglion.

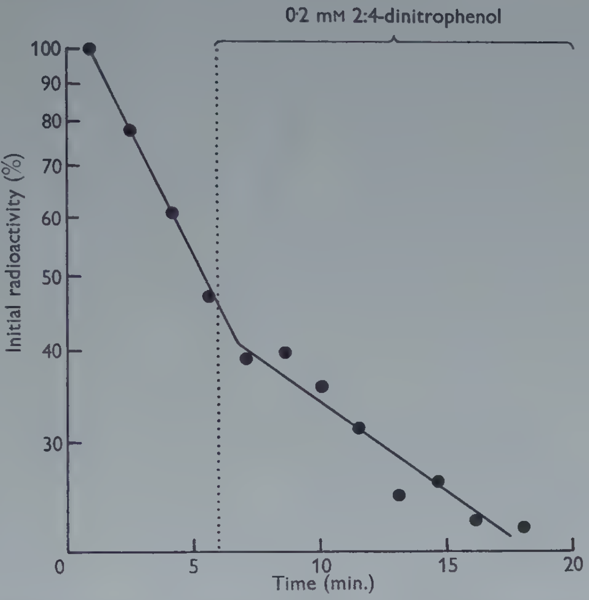

Fig. 3. The effect of 0·2 mM./l. 2:4-dinitrophenol on the escape of ^{24}Na from an isolated terminal abdominal ganglion.

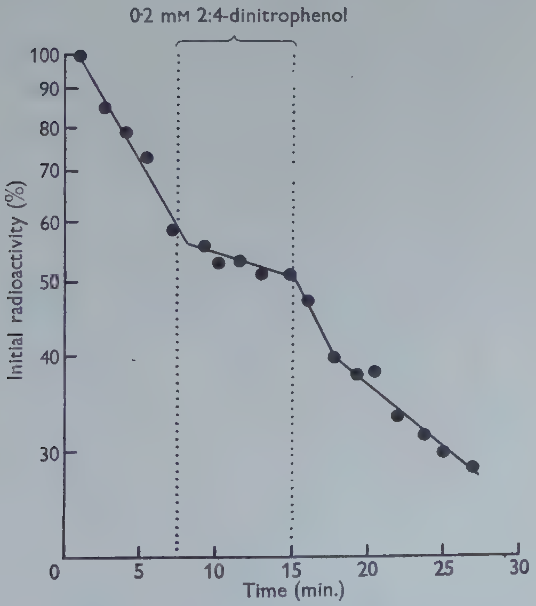

Fig. 4. The effect of 0·2 mM./l. 2:4-dinitrophenol followed by a return to normal physiological solution on the rate of loss of ^{24}Na from the terminal abdominal ganglion.

Table 3. *The effect of* 0·2 mM./l. 2:4-*dinitrophenol on the half time of sodium loss from normal and desheathed ganglia*

Preparation	Serial	Initial $t_{0\cdot5}$ (min.)	Poisoned $t_{0\cdot5}$ (min.)	Ratio $\frac{t_{0\cdot5}\text{ poisoned}}{t_{0\cdot5}\text{ initial}}$	Mean
Normal	1	4·9	17·4	3·7	2·8
	2	11·8	36·0	3·0	
	3	5·5	11·7	2·2	
	4	4·5	11·6	2·6	
Desheathed	5	4·0	8·0	2·0	1·8
	6	3·2	7·0	2·2	
	7	4·6	6·8	1·5	
	8	4·1	6·7	1·6	
	9	4·6	8·5	1·8	

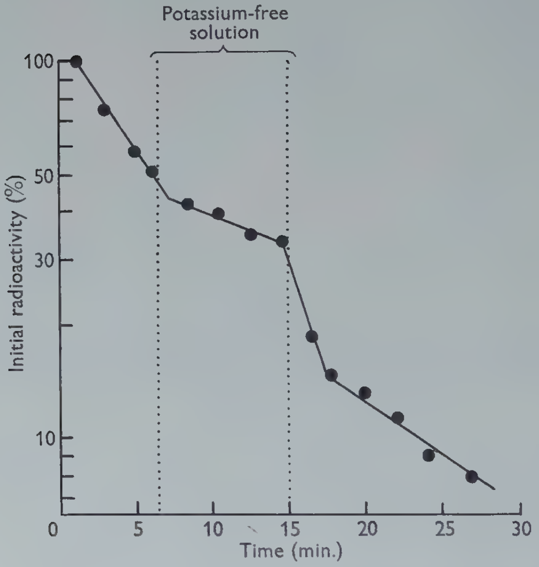

Fig. 5. The effect of potassium-free solution on the rate of loss of ^{24}Na from an isolated abdominal ganglion.

Table 4. *The effect of potassium-free solution on the rate of loss of sodium ions from normal and desheathed ganglia*

Preparation	Serial	Initial $t_{0\cdot5}$ (min.)	K-free $t_{0\cdot5}$ (min.)	Ratio $\frac{t_{0\cdot5}\text{ K-free}}{t_{0\cdot5}\text{ initial}}$	Mean
Normal	1	6·5	13·2	2·0	2·2
	2	7·4	18·0	2·4	
	3	4·9	9·5	1·9	
	4	4·6	12·8	2·8	
	5	4·2	8·3	1·9	
Desheathed	6	4·2	6·3	1·5	1·6
	7	4·4	8·3	1·9	
	8	4·5	6·2	1·4	
	9	5·0	7·5	1·5	
	10	4·0	6·5	1·6	

The point of contrast between the effluxes from the terminal ganglion and from the whole nerve cord used in the previous investigation was the apparent absence, in the case of the isolated ganglion, of a final slow phase of sodium loss in a region of low radioactivity. In the previous study (Treherne, 1961*b*) this phase was tentatively identified with the breakdown of the normal sodium extrusion mechanism in the isolated nerve cord when separated from its tracheal supply. Thus according to this hypothesis it could be postulated that in the present experiments the isolation of the ganglion resulted in a less serious interference with the normal metabolism so that the breakdown of sodium extrusion did not occur until later at a very low level of activity beyond the limits of this technique.

The present results have shown that, as in the whole abdominal nerve cord (Treherene, 1961*b*), the rate of loss of sodium was apparently an active process which was slowed down by the presence of 2:4-dinitrophenol at relatively low concentration. Similarly the extrusion of sodium in the terminal ganglion was reduced in the potassium-free solution, demonstrating a linkage of potassium influx with sodium efflux.

The rate of efflux of sodium ions from the terminal abdominal ganglion was not significantly affected by the removal of about 50% of the connective tissue and cellular sheath. On the basis of these results it must be concluded, therefore, that the rate-limiting process in the efflux of sodium measured by this technique was not the transfer of ions across the cellular perineurium. In addition it follows from this that the diffusion of sodium ions through the connective tissue sheath must also have occurred relatively rapidly, a result which had been previously predicted (Treherne, 1961*a*; Wigglesworth, 1960). The rate-limiting process measured in these experiments must, therefore, be associated with some components of the central nervous system lying at a deeper level than the perineurium. Perhaps the most obvious possibility is that the efflux of ^{24}Na measured in these experiments was, in fact, the result of the transfer of sodium ions across the cell membranes of the underlying tissues. In this case the similarity of the $t_{0.5}$ between the connectives and the terminal ganglion becomes explicable, for under these circumstances the efflux might be expected to be independent of the surface/volume ratio of the whole organ.

The results described above do not, of course, give any definite information about the nature of the processes involved in the passage of ions across the perineurium. However, the fact that the presence of dinitrophenol and potassium-free solution appeared to have slightly less effect on sodium efflux in the desheathed preparations might suggest that this layer of cells perhaps plays more than a passive role in the ionic regulation of the central nervous system of this insect.

The addition of poison to, or the omission of potassium ions from, the external solution has been shown to produce a fairly rapid slowing down of sodium extrusion from the abdominal nerve cord. The fact that the rate-limiting process is not, apparently, the penetration of the superficial perilemma implies that these changes in the chemical composition of the bathing solution are quickly transmitted to the deeper layers of the central nervous system. This conclusion is perhaps rather unexpected in view of the appreciable delay in the breakdown of normal electrical activity obtained when the insect nervous system was exposed to solutions of high potassium concentration (Hoyle, 1953; Twarog & Roeder, 1956).

In some previously published accounts on the entry of ^{42}K and ^{24}Na into the intact abdominal nerve cord of *Periplaneta* (Treherne, 1961 *a*, *c*) an attempt was made to calculate the fluxes of these ions between the haemolymph and the central nervous system. These ionic movements were calculated with the conventional equations used to describe fluxes in cells and tissues. This procedure involved the assumption that the rate-limiting process was the transfer across the superficial boundary and that the movements within the underlying layers occurred rapidly so that the labelled ions were effectively well mixed. The present results have shown that these assumptions represented an oversimplification and consequently the calculated values have little significance. It is hoped that in a future investigation the fluxes taking place between the central nervous system and the haemolymph can be calculated for this more complex system.

SUMMARY

1. The rate of loss of ^{24}Na from the terminal abdominal ganglion of *Periplaneta americana* L. has been studied by measuring the decline in radioactivity associated with an isolated preparation maintained in flowing physiological solution.

2. The rate of sodium efflux was substantially reduced in the presence of 0·2 mM./l. dinitrophenol and in potassium-free solution.

3. The extrusion of ^{24}Na was not significantly affected by the removal of the fibrous and cellular sheath surrounding the ganglion. The rate-limiting process in the efflux of sodium measured in the experiments was not, therefore, the transfer of ions across the nerve sheath, but an extrusion from tissues lying at a deeper level in the central nervous system.

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THE KINETICS OF SODIUM TRANSFER IN THE CENTRAL NERVOUS SYSTEM OF THE COCKROACH, *PERIPLANETA AMERICANA* L.

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INTRODUCTION

Some previous investigations have shown that the exchanges of sodium and potassium ions between the haemolymph and the cockroach central nervous system occurred relatively rapidly (Treherne, 1961*a*) and appeared to be effected by a mechanism involving an active extrusion of sodium ions (Treherne, 1961*b*). More recently it has also been shown that the measured efflux of sodium ions was not significantly affected by the removal of substantial portions of the cellular and fibrous nerve sheath (Treherne, 1961*c*). It was concluded from this that the rate-limiting factor measured in these experiments was not the transfer of ions across the perilemma but the extrusion of sodium from the underlying tissues of the central nervous system. Thus any rate-limiting movements of ions across the perilemma occurred too rapidly to be measured by the techniques used in the previous investigations. In the present experiments, therefore, an attempt has been made to measure the rapid component of ^{24}Na exchange by determining the rate of loss of radioactivity obtained on washing isolated nerve cords and single connectives and ganglia for relatively short periods in successive volumes of physiological solution.

METHODS

The experiments described in this paper were carried out using the abdominal nerve cords of adult male *Periplanata americana* L. In these experiments the nerve cords were made radioactive by soaking them for varying periods in a solution containing ^{24}Na (0·1–0·5 mc./ml.). With short loading periods (20 sec.–5·0 min.) the isolated ligatured nerve cords were soaked in the oxygenated physiological solution; for longer loading periods (5–20 min.) the nerve cords of decapitated individuals were perfused with the radioactive solution as described in a previous paper (Treherne, 1961*c*). The ligatures were tied with threads pulled from 15 denier nylon stockings. The composition of the radioactive solution used was that given by Treherne (1961*a*). On removal from the radioactive solution the ligatured nerve cords were carefully blotted and then washed for varying periods in successive 0·2 ml. amounts of inactive solution of the same composition. The amount of ^{24}Na remaining in the nerve cord at varying times was determined from the measured radioactivity of the washings. The radioactivity measurements were made with a Mullard MX 123 G.M. tube linked to a 100 c. Panax scaler.

Some preliminary measurements were made to estimate the extent of any 'inulin space' in the central nervous system. This was done by soaking ligatured isolated nerve cords for 1 hr. in a 3·0% solution of ^{14}C-labelled inulin (3·0 mc./g.) made up in physiological solution. The nerve cords were then washed for 25 sec. and the ^{14}C-inulin was extracted by soaking them for 24 hr. in the physiological solution. The washing time of 25 sec. used was found to be the minimum period necessary to remove 97% of the radioactivity from the surface of a nerve cord exposed to ^{14}C-inulin for 1 sec. These values are thus likely to be minimum estimates of the 'inulin space' of this organ for some radioactivity must have leaked from within the nerve cord during the washing procedure. In a limited number of cases the rate of loss of ^{14}C-labelled inulin was determined by washing the ligatured isolated nerve cords in successive volumes of the physiological solution as for the ^{24}Na efflux experiments.

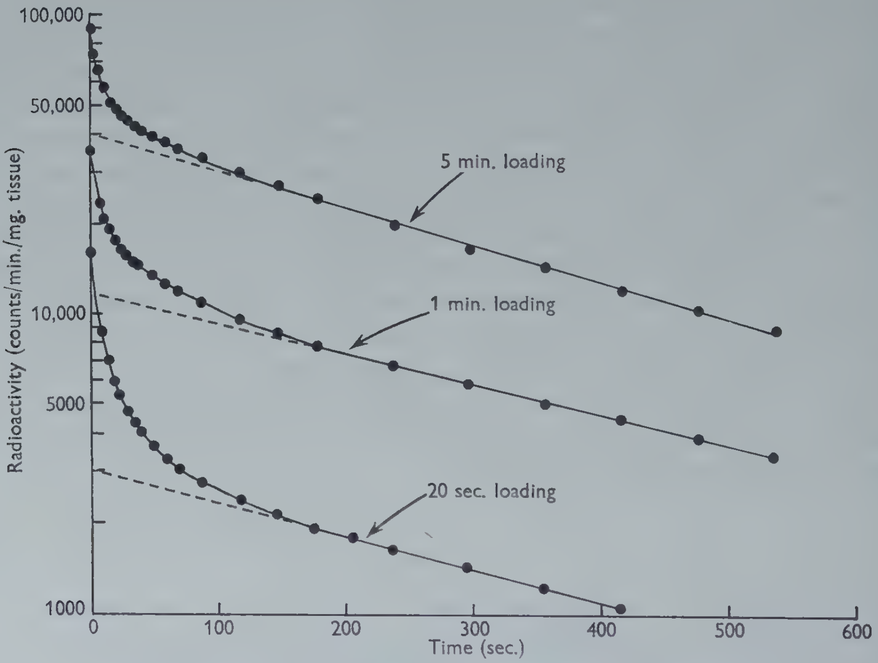

Fig. 1. A semi-logarithmic plot of the decline in radioactivity of abdominal nerve cords, loaded with ^{24}Na for varying periods, when washed in inactive solution.

RESULTS

The results illustrated in Fig. 1 show the decline in radioactivity of some isolated abdominal nerve cords, previously soaked in the solution containing ^{24}Na, when maintained in an inactive solution of the same composition. In all cases semi-logarithmic plots of the results for varying loading times appeared to follow a complex course initially, eventually assuming an exponential form after a period of between 160–200 sec.

It was found possible to separate a fast component from the curves for the loss of ^{24}Na from the nerve cords by subtraction from the initial values lying above the line extrapolated to zero time. The separation of an efflux curve into fast and slow components with data plotted semi-logarithmically with respect to time is shown in Fig. 2. The fast component illustrated in Fig. 2 was complex initially, but assumed after a few seconds a simple exponential form with a half-time ($t_{0\cdot5}$) of approximately 33·0 sec. The half-time for the slow component was, in this case, 260 sec.

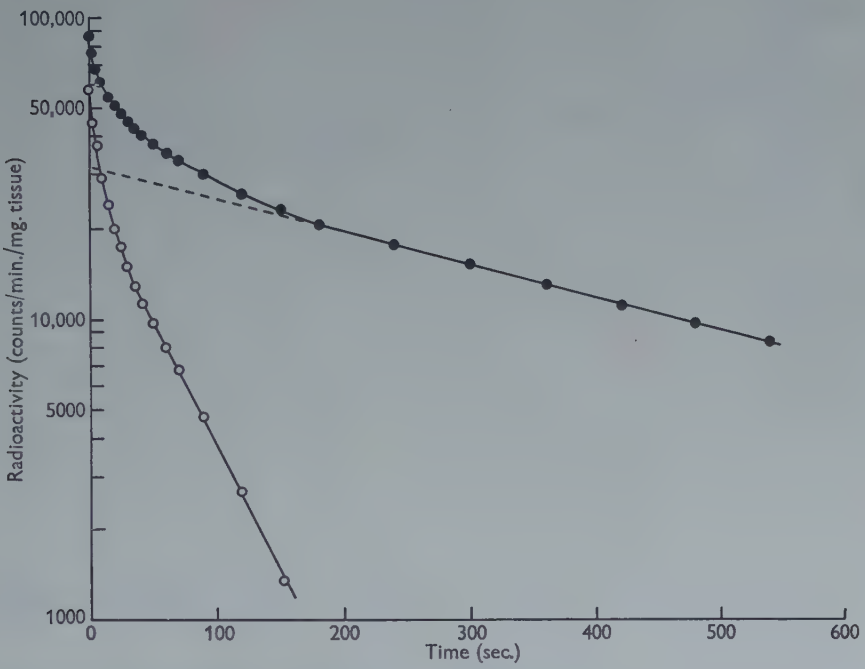

Fig. 2. The loss of ^{24}Na from a nerve cord, loaded for 10 min., when washed in inactive physiological solution (closed circles). The fast component of the main curve (open circles) was obtained by subtraction from the straight line extrapolated to zero time.

The escape of ^{24}Na from the isolated abdominal nerve cords was also measured in the presence of 0·5 mM./l. 2:4-dinitrophenol. The poison was added to the physiological solution during the initial loading period with the ^{24}Na and was present at the same concentration in the inactive solution during the subsequent efflux experiments. Previous results (Treherne, 1961*b*) have shown that there was a slight delay period of a few minutes before the poison affected the rate of extrusion of sodium from the nerve cords. In the present experiments, therefore, the nerve cords which were loaded with ^{24}Na for only short periods (less than 5 min.) were pretreated with 0·5 mM./l. dinitrophenol to maintain a constant exposure to the poison of 5 min. before the efflux experiments were commenced. Fig. 3 shows the escape of ^{24}Na from a poisoned preparation loaded with ^{24}Na for 10 min. In this experiment the fast component was not abolished by the presence of the poison, in fact $t_{0\cdot5}$ in this case was 33·0 sec., which was the same as that for the normal preparation illustrated in Fig. 2. In this particular experiment the slow component for ^{24}Na efflux was, however, much reduced as compared with the normal preparation. The effects of 0·5 mM./l. 2:4-dinitrophenol on the escape of ^{24}Na from the isolated nerve cords are summarized in Table 1. The results clearly indicate that the presence of the poison affected the slow phase of sodium loss but not the initial fast component.

The total activity of the ^{24}Na in the slowly exchanging fraction was estimated by extrapolation of the slow component to zero time. Fig. 4 illustrates the estimated

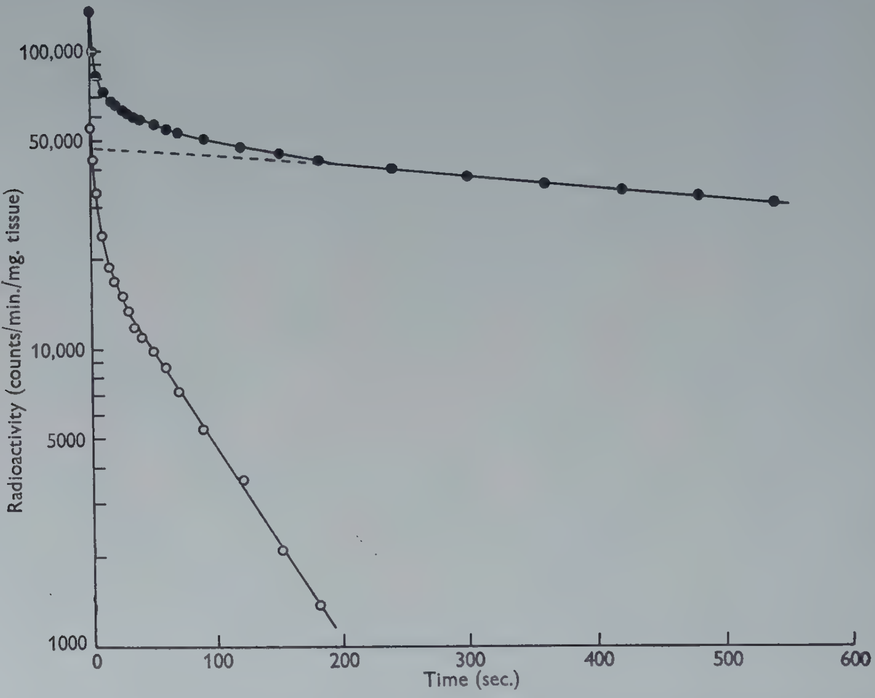

Fig. 3. The escape of ^{24}Na from a nerve cord in inactive solution in the presence of 0·5 mM./l. 2:4-dinitrophenol. The open circles represent the fast component obtained by subtraction from the straight line (closed circles) extrapolated to zero time.

Table 1. *The half-times for the fast and slow components of* ^{24}Na *escape from normal and poisoned abdominal nerve cords*

Experimental conditions	Loading time (sec.)	Fast component $t_{0.5}$ (sec.)	Slow component $t_{0.5}$ (sec.)
Normal	20	22	278
	30	31	264
	60	32	293
	120	23	240
	300	26	240
	600	33	260
	1200	32	360
	Mean	28·4	277·0
0·5 mM./l. 2:4-dinitrophenol	20	31	486
	30	34	498
	60	30	580
	120	36	648
	300	30	546
	600	33	592
	1200	27	800
	Mean	31·5	593·0

radioactivity of the slowly escaping fraction at varying times after exposure to the solution containing ^{24}Na. These data would appear to show that the poison had little effect on the rate of accumulation of the radioactive ions in the slowly exchanging fraction. The results are, however, too few to judge the equilibrium level of radioactivity as between the normal and poisoned preparations.

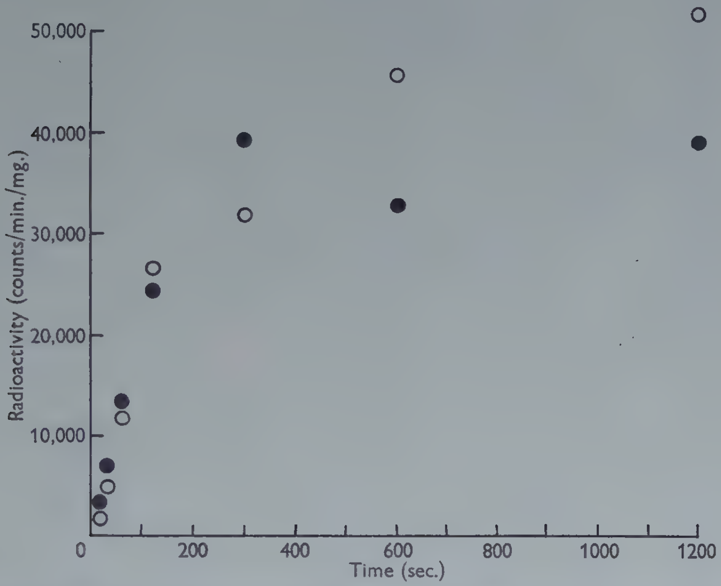

Fig. 4. The estimated activity of the slowly exchanging fraction of the ^{24}Na plotted against time for varying loading periods. The closed circles represent preparations in normal physiological solution; the open circles those in a solution containing 0·5 mM./l. 2:4-dinitrophenol.

The escape of ^{24}Na from isolated ligatured fragments of the central nervous system was studied in some experiments. The loss of radio-sodium from the terminal abdominal ganglion and from the connective between the fourth and fifth abdominal ganglia was found to occur as a two-stage process as for the whole abdominal nerve cord. The results for the escape of ^{24}Na from a single isolated connective are illustrated in Fig. 5. In this case, as for the whole nerve cord, the fast component appeared to be initially complex eventually becoming a simple exponential function. The half-times for ^{24}Na loss were in this experiment: 41·0 sec. for the fast phase and 338·0 sec. for the slow.

The effect of removal of a substantial portion of the perilemma on the rate of loss of sodium was studied in this investigation, by comparison of sodium loss as between an intact isolated terminal abdominal ganglion and one in which the dorsal portion of the cellular and connective tissue sheath was removed with sharpened watchmaker's forceps. Fig. 6 illustrates the fast components for the escape of ^{24}Na from normal and partially desheathed ganglia. The effects of removal on ^{24}Na loss were not obvious, certainly the final exponential phase was not significantly affected by the desheathing. Any effect of this procedure must, therefore, be sought in the initial complex phase which is at the moment very difficult to analyse, especially as the initial portion will also be affected by varying amounts of surface radioactivity.

Preliminary experiments were carried out in an attempt to discover the extent of any extracellular space in the central nervous system of this insect. This was done by the conventional method of measuring the inulin space. ^{14}C-labelled polysaccharide was used. Table 2 shows the inulin space as a percentage of nerve-cord water in washed nerve cords previously soaked for 1 hr. in the radioactive solution.

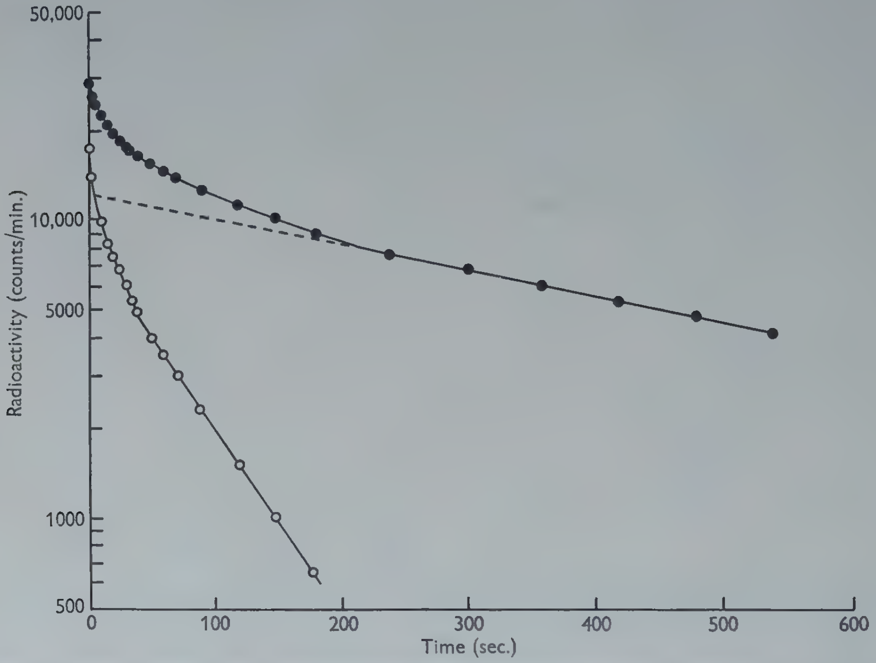

Fig. 5. The fast (open circles) and slow components (closed circles) for the efflux of ^{24}Na from a single connective when washed in inactive solution. The connective was taken from a nerve cord previously soaked for 5 min. in the radioactive solution.

The movements of the ^{14}C-labelled inulin molecules within spaces demonstrated in the previous experiments was studied by determining the rate of loss of radioactivity from the abdominal nerve cord. Fig. 7 shows the measured radioactivity associated with the nerve cord plotted against time when the preparation was washed in inactive physiological solution. These data when plotted logarithmically showed an initial curved portion which eventually gave way to an exponential decline, with a half-time of 214·0 sec.

DISCUSSION

The results outlined above showed that the escape of ^{24}Na from the isolated abdominal nerve cord occurred as a two-stage process—an initial rapid phase, with a half-time of about 28·5 sec., eventually giving way to a slow component, with a half-time of approximately 277·0 sec. The experiments with dinitrophenol clearly indicated that only the slow phase of sodium efflux was reduced by the presence of a metabolic inhibitor, the initial rapid phase being unaffected. The slow phase of sodium loss demonstrated by the present technique thus corresponds both qualitatively and quantitatively with the outward movement of sodium ions from the central

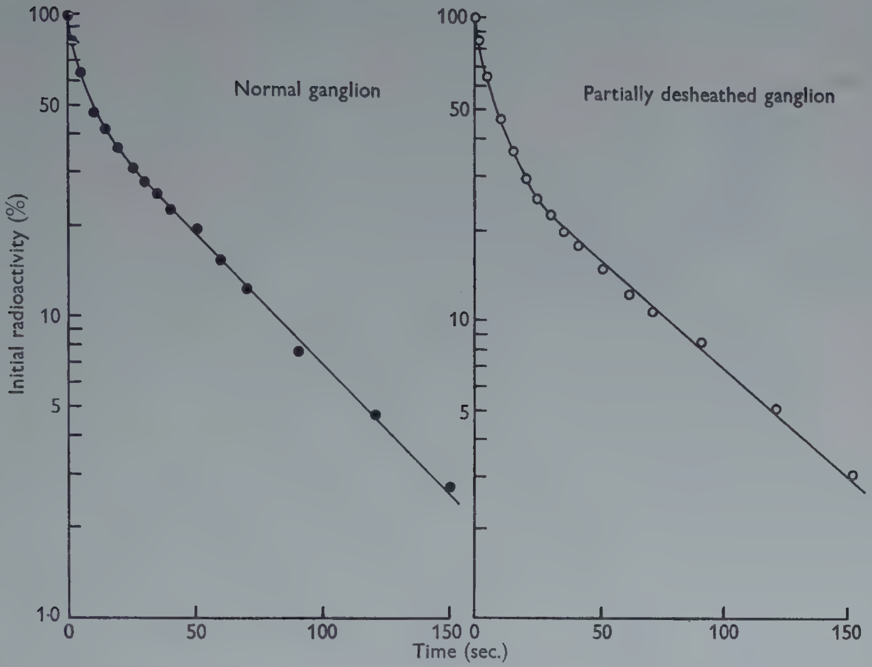

Fig. 6. The fast components obtained in experiments on the escape of ^{24}Na from intact and partially desheathed terminal abdominal ganglia washed in inactive physiological solution.

Table 2. *The inulin space as a percentage of the nerve-cord water, measured in washed preparations*

Serial	Inulin space (%)	Mean
1	9·4	10·1 %
2	13·1	
3	9·6	
4	13·6	
5	6·8	
6	7·9	

nervous system measured in previous investigations (Treherne, 1961*b*; 1961*c*). In the earlier studies in which the radioactive nerve cords were suspended in flowing saline above a GM tube, the technique was not capable of measuring the extremely rapid initial escape of ^{24}Na demonstrated in the present investigation. In these earlier studies then the fast phase of sodium efflux was not recognized.

The question which now has to be considered is the identity of the sodium ions contained in the rapidly exchanging fraction in the central nervous system. The amount of the rapidly exchanging sodium would seem to be too great to attribute this fraction to ^{24}Na associated with the surface of the abdominal nerve cord. For example, in the experiment in which the nerve cord was loaded for 10 min. the radioactivity in the rapidly exchanging fraction would, assuming it to be at the same concentration as in the external solution, account for some 30% of the nerve-cord water. It seems reasonable to suppose then that a substantial proportion of the rapidly exchanging

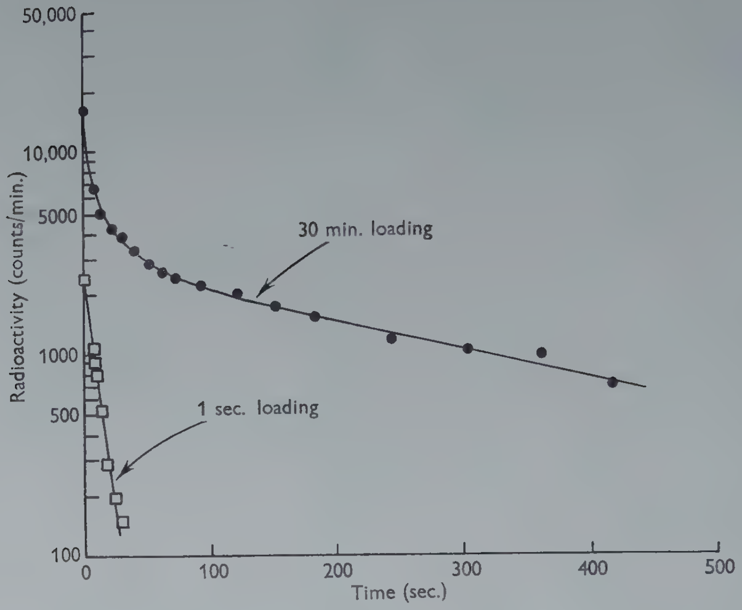

Fig. 7. The escape of ^{14}C-inulin from a nerve cord soaked for 1 sec. and for 30 min. in a 3·0% radioactive solution.

sodium fraction is contained within the central nervous system. This supposition is supported by a consideration of the processes involved in the diffusion of ions and molecules through the spaces in a mass of tissue. According to Hill (1928) diffusion from the extracellular space in a cylindrical muscle will be initially complex but will eventually follow a simple exponential course with a half-time given by

$$t_{0\cdot5} = 0\cdot118\, r_0^2/D',$$

where r_0 is the radius of the muscle and D' the diffusion constant in the extracellular spaces. This equation can thus be applied to the data for the loss of ^{24}Na from the single isolated connectives used in the present investigation, in which the average $t_{0\cdot5}$ was 43·0 sec. and r_0 averaged 0·145 mm. The value for D' thus becomes $5\cdot77 \times 10^{-7}$ cm.^{2}sec.$^{-1}$, which is approximately one-thirtieth of that for the free diffusion of sodium ions. Such a reduction in the apparent diffusion coefficient could be due, as Harris & Burn (1949) point out, to the increase in the effective path length for ions diffusing between a complex collection of cellular structures. Apparent reductions of between one-eighth and one-sixtyfifth have been noted for the diffusion of sodium ions in the extracellular spaces in cat nerves (Krnjevic, 1955) which is of the same order as for the central nervous system of this insect.

The data for the escape of sodium ions from the central nervous system is thus not inconsistent with the hypothesis that the rapidly exchanging sodium fraction represents the ions contained in extracellular spaces. The experiments with the ^{14}C-inulin demonstrated the existence of a space accessible to the polysaccharide molecules which would occupy at least about 10% of the nerve-cord water. The diffusion of the inulin molecules within the extracellular space can also be compared with that for the loss of ^{24}Na from the abdominal nerve cord. Hill's equation, given above, can be modified for a complex structure such as the abdominal nerve by representing it thus

$$t_{0\cdot5} = a\frac{1}{D'}$$

where a is a constant. It follows from this that

$$\frac{t_{0\cdot5\,(\text{Na})}}{t_{0\cdot5\,(\text{inulin})}} = \frac{D'_{(\text{inulin})}}{D'_{(\text{Na})}} = \frac{D_{(\text{inulin})}}{D_{(\text{Na})}},$$

where D' is the apparent diffusion constant in the extracellular spaces and D the free diffusion constant. Thus from the experimental data

$$\frac{t_{0\cdot5\,(\text{Na})}}{t_{0\cdot5\,(\text{inulin})}} = \frac{28\cdot5}{214\cdot0} = 0\cdot133$$

and

$$\frac{D_{(\text{inulin})}}{D_{(\text{Na})}} = \frac{0\cdot16\times10^{-5}}{1\cdot26\times10^{-5}} = 0\cdot127.$$

The two values obtained from these ratios agree reasonably well, which is additional evidence that the fast component in sodium efflux was, in fact, a movement within the extracellular space demonstrated by the ^{14}C-inulin.

The demonstration of a rapid exchange of sodium ions between an extracellular space in the central nervous system and the external solution helps to clarify the results obtained in a previous investigation (Treherne, 1961*c*). In this earlier study it was found that the rate of sodium extrusion from the isolated terminal abdominal ganglion was not significantly affected by the removal of substantial portions of the cellular and fibrous nerve sheath. It has now been shown that the efflux of ^{24}Na measured in the previous experiments corresponded to the slow phase of sodium loss obtained in the present experiments and, in fact, represents an extrusion from the cellular components of the central nervous system. Thus as the relatively slow extrusion takes place into an extracellular space from which ions escape very rapidly it is not surprising that in the previous study removal of the perilemma did not affect the measured loss of sodium ions.

The exchange of sodium ions taking place between the extracellular space and the various cellular components of the cockroach central nervous system appeared to be essentially similar to those taking place in cephalopod giant axons (cf. Hodgkin, 1958). As in the squid axon the accumulation of radioactive sodium within the various cells of the central nervous system of the cockroach appeared to be a passive process, unaffected by the presence of a metabolic inhibitor. The efflux of sodium from the cellular components of the central nervous system was, however, reduced in the presence of metabolic inhibitors as in the cephalopod giant axon. In the absence of external potassium ions (Treherne, 1961*b*, 1961*c*) the extrusion of sodium ions from the cellular components was also reduced indicating that, as in the squid axon, the efflux is effected by some sort of linked sodium pump.

The demonstration of the rapid exchanges of sodium ions between the external solution and an extracellular space in the central nervous system of this insect is perhaps rather unexpected in view of the observations of Hoyle (1953) and of Twarog & Roeder (1956) that the blocking time for insect nerve is dramatically reduced in solutions of high potassium concentration when the perilemma is removed. Twarog & Roeder, for example, demonstrated that in the intact cockroach nerve cord the blocking time in a solution containing 140 mM./l. potassium was between 22–30 min., as compared to 60–90 sec. in the desheathed preparation. The results have been generally interpreted as indicating that the perilemma functions as a diffusion barrier of some kind. The present experiments have demonstrated, however, an efflux from an extracellular space through the nerve sheath which appears to be a purely passive process and which takes place relatively rapidly ($t_{0 \cdot 5} = 28 \cdot 5$ sec.). It is hoped that some further researches on the cockroach central nervous system may help to throw some light on this apparently paradoxical situation.

SUMMARY

1. The exchange of sodium ions in the cockroach central nervous system has been studied by following the escape of ^{24}Na from isolated abdominal nerve cords, single connectives and ganglia. Particular attention was paid to the initial rapid exchanges of sodium.

2. The escape of sodium ions occurred as a two-stage process, an initial rapid phase eventually giving way to a slower exponential phase of sodium loss. The fast phase of efflux was not affected by the presence of 2:4-dinitrophenol, although this poison significantly reduced the second slow phase of sodium extrusion.

3. The initial fast phase is attributed to a rapid diffusion from an extracellular space, demonstrated by ^{14}C-inulin; the second phase is identified as the slower extrusion from the cellular components of the central nervous system.

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VISCO-ELASTIC PROPERTIES OF THE TUNICA EXTERNA OF THE SWIMBLADDER IN CYPRINIDAE

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INTRODUCTION

The anterior sac of the swimbladder in the Cyprinidae is composed of two layers, the tunica externa and the tunica interna, which are connected to each other only weakly by a layer of loose connective tissue. A slit runs in the anterior dorsal mid-line of the tunica externa. The most posterior of the Weberian ossicles, the tripodes, are attached to the edges of this slit. This arrangement, which was demonstrated, apparently independently, by Evans (1925) (with some errors) and Chranilov (1929), is illustrated in Fig. 1.

Chranilov (1929) suggested that as the tissue connexion between tunica externa and tunica interna is so slight, small sliding movements of the tunicae relative to each other should occur readily. Hence, an increase in the volume of the anterior sac will result in outward movement of the edges of the slit, rotating the tripodes forward. On a subsequent decrease in volume, the edges of the slit and the tripodes will be returned to their original positions by the elastic recoil of the ligaments labelled 1 and 2 in Fig. 1. Further, if one or both of these ligaments is of such a length as to maintain tension in ligaments 3 and 4, movements of each tripus will be transmitted via the intercalarium to the scaphium, which forms one wall of the atrium sinus impar. Changes in volume of the anterior sac will thus result in displacement of the contents of the sinus impar. It has been shown that the Weberian apparatus is of value to Cyprinidae in detecting changes of anterior sac volume due both to sound waves (von Frisch & Stetter, 1932) and to slow changes of ambient pressure, such as the fish will experience if its depth below the water surface changes (Dijkgraaf, 1941). The extent to which the tripodes move for a given change of anterior sac volume will depend on the relationship between the elastic properties of the tunica externa and of the ligaments 1 and 2.

Progressive decreases of anterior sac volume will cease to cause movement of the Weberian ossicles as the ligaments 1 and 2 become slack, while progressive increases will cease to cause movement of the scaphia as the ligaments 3 and 4 become slack. It would thus appear that the ears receive information only of those changes of anterior sac volume which lie within a certain range.

A very small change of depth will produce a change of anterior sac volume as great as the amplitude of volume changes resulting from an extremely loud sound. There is a possibility that a quite small change of depth might result in a change of anterior sac volume to a value beyond the range within which the Weberian ossicles operate.

Such a change of depth would thus produce partial deafness until the quantity of gas in the swimbladder was adjusted. This would plainly be undesirable. It might be avoided, however, if an appropriate high-pass frequency filter was built into the Weberian apparatus.

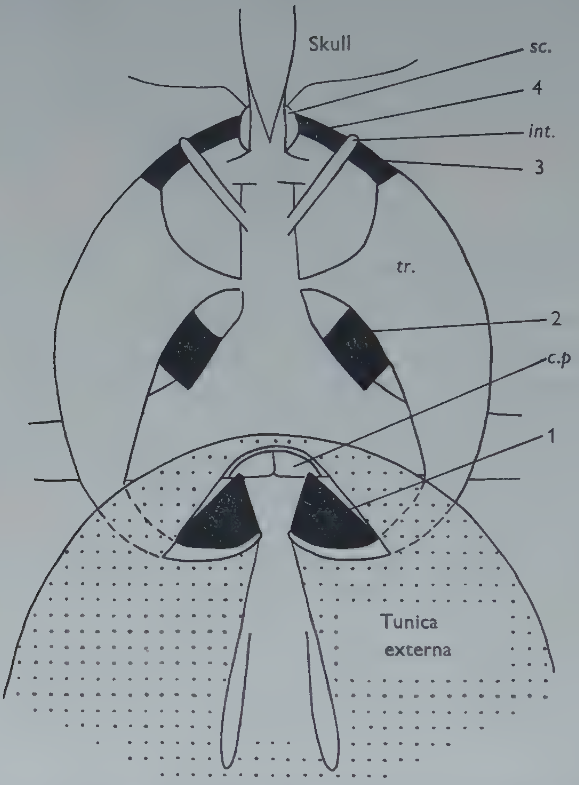

Fig. 1. The Weberian apparatus of a typical cyprinid. The diagram represents a dissection, seen in ventral view, with the ventral half of the tunica externa and the whole of the tunica interna removed. *c.p.*, central plate; *tr.*, tripus; *int.*, intercalarium; *sc.*, scaphium; 1, 2, 3, 4, ligaments.

The tunica externa consists (Fauré-Fremiet & Garrault, 1937) of a trellis of elastic fibres, and needles of ichthyocol, which is a form of collagen consisting of short needles (length up to *ca.* 0·3 mm.) instead of long fibres. Ichthyocol slowly becomes fibrous after removal from the animal, and does so rapidly in 70% alcohol. It can therefore be examined only in fresh preparations, after teasing or after digesting the elastic fibres in pancreatin.

The only continuous elements in the tunica externa are thus elastic fibres, which are highly extensible (Burton, 1954). The ichthyocol, while unable to contribute to the elasticity of the tunica externa, might give it high viscosity. If this were so, the tunica externa would be rather inextensible to oscillating forces, such as it would be subject to when the swimbladder vibrated at audio frequencies, but highly extensible to forces applied over relatively long periods, such as those resulting from a change of swimbladder volume due to a change of depth. Provided that ligaments 1 and 2 were less viscous than the tunica externa, slow changes of swimbladder volume would result in smaller movements of the tripodes than rapid movements of the same amplitude. The Weberian apparatus would then incorporate a high-pass frequency filter, such as was suggested above. This paper is mainly concerned with the visco-elastic properties of the tunica externa.

Experiments on the tunica interna will also be reported. It appears to consist entirely of smooth muscle and ordinary collagen, without ichthyocol or elastic fibres.

The position of the slit makes it impossible to cut a ring of tunica externa (whose visco-elastic properties might be determined by stretching between hooks) such that elastic fibres run parallel to the cut edges, and so continuously round it. A method was therefore devised, based on the following principle.

A piece of the swimbladder layer under investigation was clamped as a membrane over a circular aperture. A pressure difference was applied across the membrane and stretched it into a dome-like shape. The extent of this bulging was determined at a series of measured times after the application of the pressure difference.

MATERIAL AND METHOD

Most of the experiments described in this paper were carried out on tench (*Tinca tinca* (L.)). A few experiments on mirror carp (*Cyprinus carpio* L.) and rudd (*Scardinius erythrophthalmus* (L.)) are also reported. Most of the fish used weighed between 100 and 250 g.

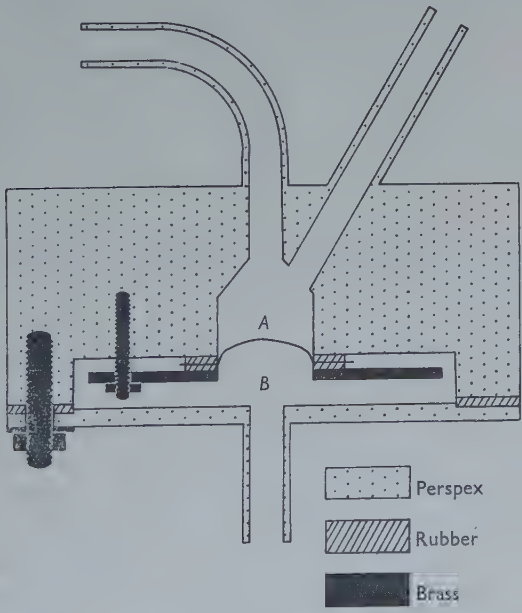

Fig. 2. The membrane holder.

The principal part of the apparatus is shown in section in Fig. 2. It is a hollow cylinder of Perspex of diameter 3 in., entered axially above and below by Perspex tubes, and obliquely by another tube from above. A brass plate with a central hole is held by three bolts, symmetrically placed, of which only one appears in the section; it is used to clamp a membrane (either tunica externa or tunica interna) between rubber washers so as to separate cavity *A* from cavity *B*. The washers are cut from a toy balloon, and are lightly greased. Access to the interior of the apparatus is provided by the removable bottom, which is held in position by three bolts, symmetrically placed, of which only one appears in the section.

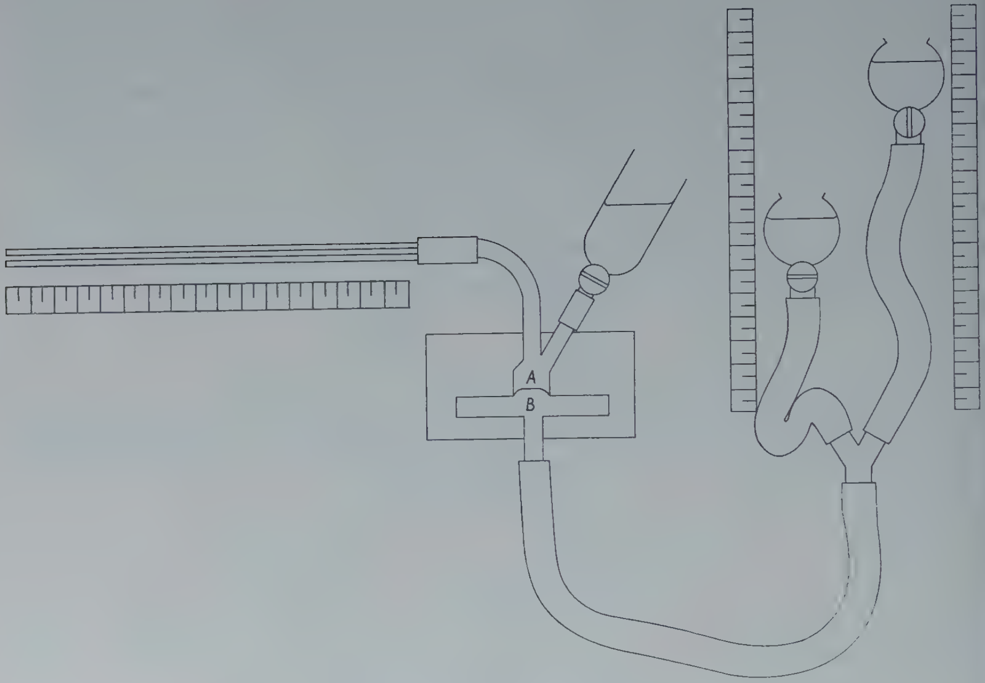

Fig. 3. The complete apparatus.

The complete apparatus is represented in Fig. 3. It is filled with Ringer solution (Young, 1933; formula recommended for freshwater teleosts other than eels) whose surfaces are shown in the funnels, and which forms a meniscus in the thick-walled capillary. The position of this meniscus can be adjusted by means of the funnel opening through the oblique tube into cavity *A*.

The other two funnels are held by retort stands, and their heights can be adjusted. Either may be used to provide a head of water to give a pressure difference between *A* and *B*. The provision of two funnels makes possible instantaneous changing of the pressure by simultaneously turning one tap off and the other on. The pressure difference between *A* and *B* is given by the height above the capillary of the Ringer surface in the funnel whose tap is open. It stretches the membrane separating the cavities, which bulges into cavity *A*. This results in movement of the meniscus along the capillary. As the bore of the capillary has been determined by weighing the mercury which filled a measured length of it, this movement gives a measure of the extent to which the membrane has been stretched. The time course of an extension can be followed.

The diameter of cavity *A* is 0·97 cm. Capillaries of capacity about 0·01 ml./cm. have been found convenient: they are fine enough to give reasonable sensitivity, and coarse enough to give a response which is, for practical purposes, instantaneous.

All experiments were started with a pressure difference of 4 cm. water to hold the membrane taut. The rate of stretching under this low load was negligible by the time the apparatus had been set up. Care was taken to avoid subjecting the membrane to higher pressure difference while the apparatus was being set up (this was necessary as extension of the tunica externa was found to be partially irreversible). As far as possible, air bubbles were eliminated from the apparatus, but their presence could not affect the accuracy of experiments, since the recording side of the apparatus (cavity *A*) remained at constant pressure. The pressure was increased to 20, and sometimes thence to 36, cm. water.

Sometimes a leak developed in the membrane. As a check against this possibility, the pressure was briefly reversed before and after each experiment, and the consequent meniscus movement noted. This meniscus movement represented double the volume under the domed membrane. This procedure gave a measure of the total stretch that occurred in the course of the experiment, and could be compared with the value, subject to errors due to leaks, obtained by observing the movement of the meniscus through the main part of the experiment. When a leak was found to have occurred no quantitative use was made of the results of the experiment. In each of the experiments from which the data of Table 1 are derived the values for extension obtained by these two methods differed by less than 4%.

Extensions of the tunica externa were found to be partially irreversible, and it was thought necessary to consider the possibility that this result might be an artifact due to slipping of the membrane between the washers. This possibility was rejected for the following reasons:

(*a*) The effect was invariably found with tunicae externae, but in only one experiment with a tunica interna. After this experiment the tunica interna was found, as would be expected, to be wrinkled round the edges. No such wrinkling was observed in experiments on tunicae externae.

(*b*) The pressure of the washers left marks on the tunicae externae which persisted for many hours after they were removed from the apparatus. The sharpness of the edges of these marks denied the possibility of the membranes having slipped between the washers.

Experiments were carried out on both tunica externa and tunica interna of each fish, usually but not always in that order. The ventral thickening of the tunica interna was avoided. The fish was killed immediately before the first experiment, and the specimen for the second was kept in Ringer until it was required. Experiments ended between 1½ and 12 hr. after the death of the fish. There was no evidence of the deterioration of material in this period. In one case (tench D 15) experiments were carried out on two pieces of tunica externa, one immediately after the fish had been killed and the other 7 hr. later. The second piece stretched rather more rapidly than the first, but in a qualitatively similar manner.

At the large deflexions occurring in these experiments, the shape of the membrane is determined almost entirely by pure stretching, and its flexural rigidity contributes little. The standard formula for small deflexions of a uniformly loaded plate (Love, 1893, p. 199) is, therefore, quite inapplicable. An exact mathematical treatment of the relation between strain of the membrane and movement of the meniscus in the capillary would necessarily be extremely complex. As the outer margin of the membrane is fixed, less extension in the circular direction occurs peripherally than centrally. In satisfying the well-known relationship between pressure difference, tension and radii of curvature, the membrane assumes the form of a dome which rises sharply at the edges but is more flattened centrally.

On this account the extending membrane may be considered as forming approximately the surface of a low cylinder open at one end. The radius of the cylinder, r, is constant and equal to the radius of the hole across which the membrane is clamped. Its height will be zero when there is no pressure difference across the membrane: let it be h after equilibration at a pressure difference p, and $(h+\Delta h_t)$ a period of time, t, after the changing of the pressure difference to $(p+\Delta p)$.

If the thickness of the membrane is d the change in radial stress due to this change of pressure is

$$\frac{\pi r^2 \Delta p}{2\pi r d} = \frac{r \Delta p}{2d}.$$

The radial strain of the membrane at time $t = \Delta h_t/(r+h)$, the initial volume under the membrane is given by

$$V_0 = \pi r^2 h,$$

and the volume at time t

$$V_t = \pi r^2 (h+\Delta h_t),$$

whence $V_t - V_0 = \pi r^2 \Delta h_t$, and the radial strain at time $t = (V_t - V_0)/[\pi r^2 (r+h)]$. The radial strain per unit stress at time t is given by

$$\mathcal{J}_t = \frac{2d(V_t - V_0)}{\pi r^3 \Delta p (r+h)},$$

or, if h is small compared to r

$$\mathcal{J}_t \simeq \frac{2d(V_t - V_0)}{\pi r^4 \Delta p}$$

The value of $\mathcal{J}_t$ when $t = \infty$ would be the compliance* of the material of the membrane. $(V_t - V_0)$ could be calculated from the meniscus movement and the bore of the capillary. Δp and r were known. Thicknesses of tunica externa and tunica interna were measured on unfixed frozen sections of material from a 133 g. tench. Mean values of 0·18 and 0·095 mm., respectively, were found. Values of d were calculated for the tench used in the experiments on the assumption of proportionality with the cube root of weight.

It was thus possible to calculate values for $\mathcal{J}_t$.

An entirely different approximation in which the stretched membrane is assumed to take a spherical form gives values for $\mathcal{J}_t$ differing from those calculated from the above formula by only 20%. This gives added confidence that the values of compliance derived from the simple approximation here used will be accurate within a factor of two.

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* Compliance (cm.^{2}/dyne) is here used as the reciprocal of Young's modulus. This usage must be distinguished from the use of compliance (cm./dyne) as the reciprocal of stiffness. There appears to be no generally accepted term for the reciprocal of Young's modulus (Burton, 1954), but I follow Alfrey & Doty (1945) in calling this quantity 'compliance'.

RESULTS

Pieces of tunica interna of tench, rudd and mirror carp showed, in this apparatus, no appreciable viscosity. Meniscus movements were practically complete within 1 sec. of any change of pressure. Extensions were found to be completely reversible. Compliances of about 2×10^{-7} cm.^{2}/dyne were found.

In these experiments the tunicae internae were at much lower tensions than those to which they are subject in life, due to the excess internal pressure of the swimbladder (Alexander, 1959). The results obtained for them are thus of interest only as a contrast with those for tunicae externae, and in showing that the apparatus was capable of registering rapid extensions.

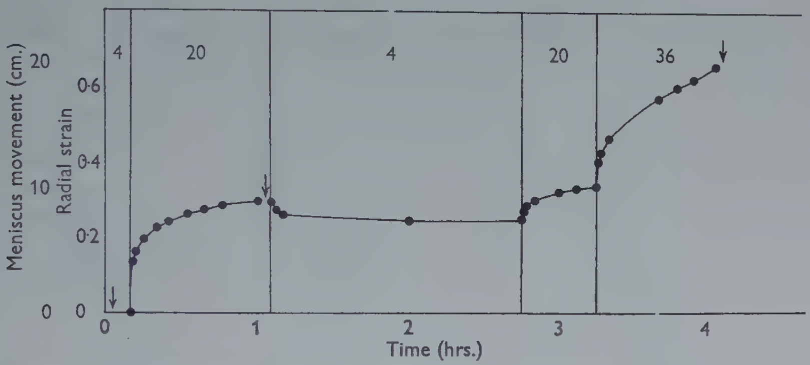

Fig. 4. Meniscus movement (cm.) and radial strain plotted against time (hrs.) for the experiment on the first of the pieces from the tunica externa of the tench D 15. The figures, 4, 20, 4, 20 and 36, give the applied pressures in cm. water; the vertical lines mark the times when these pressures were changed.

The behaviour of pieces of tunica externa was found to be considerably more complex. Fig. 4 shows the results of a typical experiment with a tench. The arrows mark the occasions when the pressure was briefly reversed, to check against the possibility of leakage through the membrane. The total strains observed were much larger than for tunica interna, but they were by no means instantaneous. The rate of stretching of tunica externa is clearly limited by a very high viscosity. The extensions were by no means completely reversible. The final rates of extension observed in each experiment were much too low to permit explanation of this irreversibility in terms of series viscous components. The course of extension can most simply be interpreted in terms of Voigt elements (i.e. of viscous components arranged in parallel with elastic ones). The partial irreversibility is behaviour of the type known in 'frozen rubbers' as described by Pryor (1950).

In Figs. 5 and 6 radial strain per unit stress, J_t, is plotted against the time t (shown on a logarithmic scale) after the initial increase of pressure from 4 to 20 cm. water in the successful experiments on the tunicae externae of tench. The values of J_t may be compared with the compliances of about 3×10^{-7} cm.^{2}/dyne which have been found for elastin (Burton, 1954). Only a small proportion, of course, of the volume of the tunica externa consists of elastin.

The distribution of gradient along such curves approximates closely to the distribution of elastic compliance with respect to retardation time, except in its tendency to suppress sharp discontinuities (Alfrey & Doty, 1945). The gradients are seen to be low for small values of t, but rise rapidly to steady values which are maintained, except in the case of D16, to the highest value of t for which measurements were made. The gradients and intercepts on the time axis of these straight parts of the curves are given in Table 1. It appears that compliance of about 8×10^{-7} cm.^{2}/dyne is associated with each increase by a factor of 10 of retardation time from about 4 to 2000 sec. or more.

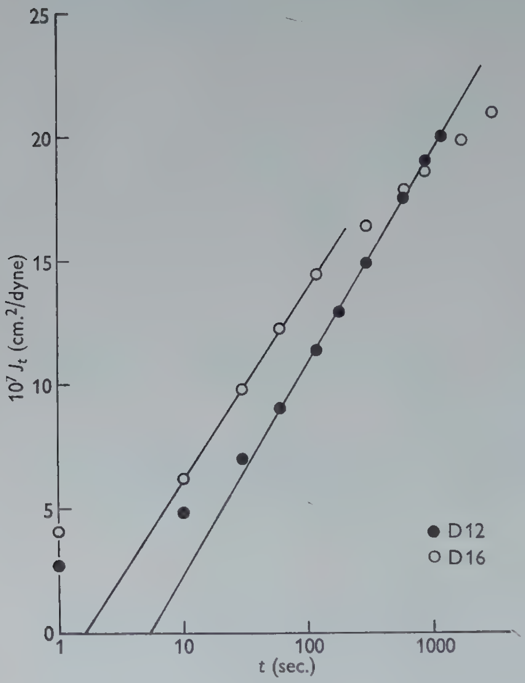

Fig. 5

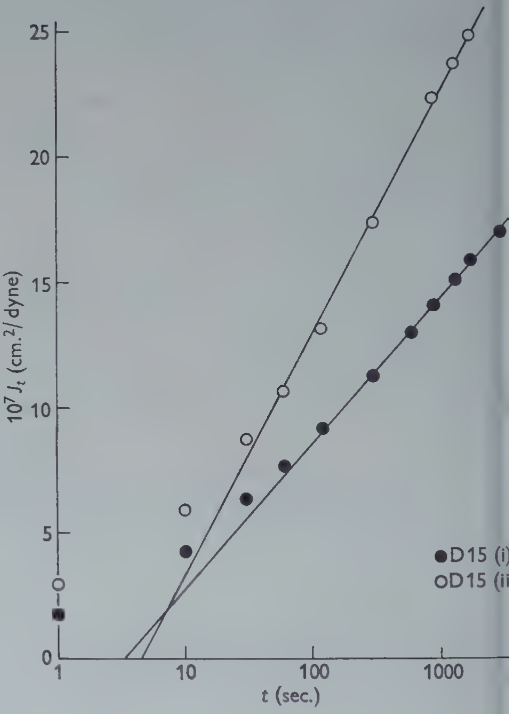

Fig. 6

Fig. 5. Radial strain per unit stress (cm.^{2}/dyne) plotted against time (in seconds, shown on a logarithmic scale) after the initial increase of pressure from 4 to 20 cm. water in the experiments on pieces of the tunicae externae of tench D12 and D16.

Fig. 6. Radial strain per unit stress (cm.^{2}/dyne) plotted against time (in seconds, shown on a logarithmic scale) after the initial increase of pressure from 4 to 20 cm. water in the experiments on pieces from the tunica externa of tench D15.

A large part of the compliance of tench tunica externa is thus associated with retardation times greater than 1 sec., and this compliance is distributed uniformly with respect to the logarithm of retardation time over a wide range of retardation times. Distributions of this type occur in rubbers and certain synthetic polymers (see, for instance, Tobolsky, Dunnell & Andrews, 1951). However, only a small part of this compliance is reversible.

In experiments D15 (both specimens of tunica externa) and D16 the pressure difference across the tunica externa was returned to 4 cm. water after a period at

20 cm. water. The course of the consequent incomplete elastic recoil was followed. The results are presented in Fig. 7, which is a graph of the same type as Figs. 5 and 6 except that meniscus movement is plotted instead of radial strain per unit stress. The latter cannot be calculated, since extension at the higher pressure was not complete when the pressure was reduced again. The restoring force is therefore not known. The meniscus movements are small, and more sensitive apparatus would be required to record them accurately. However, the data appear to show that the reversible part of the compliance of tunica externa is almost entirely associated with retardation times between 5 and 1000 sec. The mean total meniscus return, of 1·3 cm., indicates a reversible compliance of not less than $2·5 \times 10^{-7}$ cm.^{2}/dyne between the stresses involved in the experiment.

Table 1. *Gradients and intercepts on the time axis of the straight parts of the curves shown in Figs. 5 and 6.*

	Experiment				
	D 12	D 15 (i)	D 15 (ii)	D 16	Mean
Weight of fish (g.)	196	229	229	437	—
Gradient (cm.^{2}/dyne/log. cycle)	$8·5 \times 10^{-7}$	$5·7 \times 10^{-7}$	$9·6 \times 10^{-7}$	$7·6 \times 10^{-7}$	$7·9 \times 10^{-7}$
Intercept (sec.)	5·2	3·3	4·5	1·6	3·7

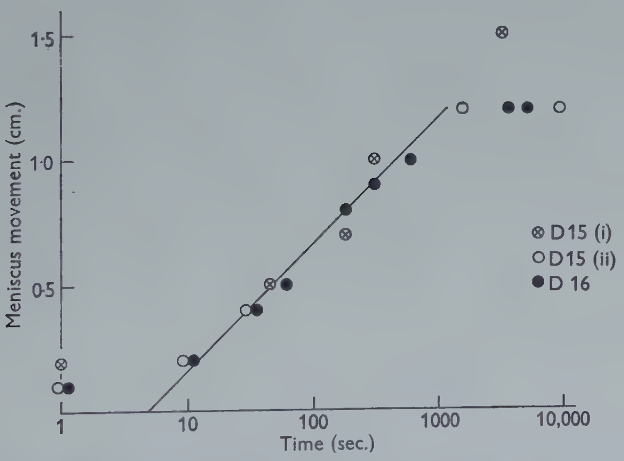

Fig. 7. Meniscus movement in cm. plotted against time (in seconds, shown on a logarithmic scale) after reduction of the pressure difference from 20 to 4 cm. water in the experiments on pieces from the tunicae externae of tench D 15 and D 16.

Experiments on the tunicae externae of two mirror carp and four rudd gave results qualitatively similar to those reported for tench, but these could not be used for quantitative analysis owing to leakage of fluid through the specimens.

Most tunicae externae used withstood pressure differences of 36 cm. water, but those of a 97 g. and a 282 g. tench tested to destruction, ruptured at 60 and 100 cm. water, respectively. Tunicae internae are much stronger.

DISCUSSION

The point was made in the introduction to this paper that the extent to which the tripodes move for a given change of anterior sac volume will depend on the relationship between the elastic properties of the tunica externa and of ligaments 1 and 2 of Fig. 1. It has been shown in this paper that the tunica externa is markedly viscous. A large part of its compliance (both of the reversible and of the irreversible components) is associated with retardation times greater than 1 sec. It will thus yield much less to forces oscillating at audio frequencies than it will over a period of many seconds to a steady force. If ligaments 1 and 2 are more normal tissues with less viscous properties, slow changes of anterior sac volume will result in smaller movements of the tripodes than oscillatory changes of the same amplitude at audio frequencies. The possibility that a change of depth will move the Weberian ossicles to the limits of their working range, and so produce temporary partial deafness, is thus reduced, without reducing the sensitivity of the system to sound.

This hypothesis depends on the viscosities of ligaments 1 and 2 being reasonably small. The viscous properties of the tunica externa are probably due to the presence of a large proportion of ichthyocol in this tissue. In sections of goldfish (*Carassius auratus* (L.)) stained with Mallory's triple stain, ligaments 1 and 2 stain (as does the tunica externa) as collagen. However, I have been unable to find ichthyocol in teased ligaments 1 and 2 of tench and crucian carp (*C. carassius* (L)). It therefore appears that this collagen is of the normal fibrous type, and there is no reason to suppose that these ligaments share the peculiar visco-elastic properties of tunica externa.

The hypothesis meets a difficulty, however, in the partial irreversibility of tunica externa extensions. One is tempted to suppose that complete reversibility may obtain in life, perhaps owing to the presence of a plasticizer. If this is not the case, the behaviour of the tunica externa will still be viscous (as shown by the results displayed in Fig. 7) but only the smaller reversible part of the compliance will be effective. The tunica externa will become permanently slack, and the Weberian apparatus will cease to function, if the anterior sac is ever allowed temporarily to exceed a certain size. However, the 'Gasspuckreflex' may be sufficient protection against this. Further investigation is required.

I find that ligaments 3 and 4 (see Fig. 1) of tench and crucian carp are rich in ichthyocol. It therefore seems likely that their visco-elastic properties may resemble those of the tunica externa. They may transmit rapid vibrations of the tripodes to the scaphia more strongly than slow movements. If so they will constitute a high-pass frequency filter supplementary to the one involving the tunica externa. An investigation of the properties of these ligaments is planned.

SUMMARY

1. The tunica externa of the anterior sac of the swimbladder in Cyprinidae is highly extensible, but a large part of its compliance is associated with retardation times greater than one second. The compliance is only partly reversible.

2. The significance of these properties is discussed in relation to a possible high-pass frequency filter for the Weberian apparatus.

I wish to thank Dr K. E. Machin for valuable advice on the physics of this problem.

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PHARMACOLOGICAL STUDIES ON THE AUDITORY SYNAPSES IN A GRASSHOPPER

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INTRODUCTION

In the nerve cord of *Gampsocleis buergeri* (*Tettigoniidae*), the auditory T large fibre lying between the brain and the metathoracic ganglion was found electrophysiologically by the present authors to receive not only an excitatory effect from the ipsilateral tympanic nerve at the prothoracic ganglion, but also inhibitory and weak excitatory effects from the contralateral one (Suga & Katsuki, 1961).

It was thus expected that the inhibitory effect was produced by inhibitory interneurons activated by the contralateral tympanic nerve. Recently the synaptic mechanism in lower animals has been revealed to some extent, and it may be very important to know in the present case what happens in these synapses. The pharmacological experiments with chemicals having specific actions on inhibitory or excitatory synapses are thought to be very profitable for the analysis of the central interaction between the tympanic nerves and the auditory T large fibres in the cord. The present paper will deal with the results which further elucidate the mechanism of interaction so far studied only by the electrophysiological technique.

The pharmacological agents used in the present work were picrotoxin, eserin, butyrylcholine, acetylcholine, GABA, γ-aminobutyrylcholine, D-tubocurarine, and strychnine. Those chemicals are known to act on synapses as follows. Picrotoxin is an excitant of excitatory synapses in the cat brain (Purpura, Girado & Grundfest, 1957). However, in crustacean stretch-receptors (Elliott & Florey, 1956; Bazemore, Elliott & Florey, 1956; Edwards & Kuffler, 1957), muscles (Van der Kloot, Robbins & Cooke, 1958; Grundfest, Reuben & Rickles, 1959; Reuben, Bergmann & Grundfest, 1959), and the central nervous system (Hichar, 1960), it is said to be an inhibitor of inhibitory synapses. Eserin suppresses the resolving action of cholinesterase on acetylcholine and enhances the acetylcholine response of a crayfish stretch-receptor (Wiersma, Furshpan & Florey, 1953). Butyrylcholine has a strong excitatory effect on a crayfish stretch-receptor and produces high-frequency discharge of impulses as acetylcholine does (Hagiwara, Kusano & Saito, 1960). GABA operates as an inhibitor on excitatory synapses in the cat brain (Purpura *et al.* 1957). However, in crustacean stretch-receptors (Bazemore, Elliott & Florey, 1956, 1957; Edwards & Kuffler, 1957, 1959; McLennan, 1957; Hagiwara *et al.* 1960) and muscle (McLennan, 1957; Grundfest *et al.* 1959; Reuben *et al.* 1959), GABA is an excitor of inhibitory synapses. γ-Aminobutyrylcholine has an effect on synapses similar to that of GABA in crayfish stretch-receptors (Hagiwara *et al.* 1960). In the cat brain D-tubocurarine is an inactivator of excitatory and inhibitory synapses and brings about the inhibition of the excitation (Purpura & Grundfest, 1956; Grundfest, 1958). Metrazol and strychnine are excitants in the central nervous system of Mammalia. However, the former excites the excitatory synapses in the same way as picrotoxin does, while the latter inhibits selectively the inhibitory synapses (Purpura *et al.* 1957). In a crayfish stretch-receptor, metrazol has an effect similar to that of picrotoxin, but only in much higher concentrations. Strychnine has, however, no effect on it (Bazemore *et al.* 1956; Elliott & Florey, 1956). Acetylcholine, and D-tubocurarine also have no effect on lobster neuromuscular synapses (Grundfest *et al.* 1959).

It seems very interesting that one and the same chemical acts on the synapses of Mammalia and Crustacea in entirely different ways, as described above. From a viewpoint of comparative neuropharmacology it is also desirable to know what events are brought about on the insect synapses which have so far not been studied.

MATERIAL AND METHOD

The animal used was *Gampsocleis buergeri*. The insect was pinned on its back on a cork board and the ventral exoskeleton covering the thoracic nerve cord was then removed. The tracheae distributing along the nerve cord were also separated from the latter. The central nervous system and the peripheral nerves were cut so that there remained only the simple nervous system consisting of the pair of tympanic nerves, the prothoracic ganglion, and both the suboesophageal-prothoracic connectives. The operated insect was put in a sound-proofed room air-conditioned at about 24° C. and the physiological saline solution[*] was applied to the nerve in order to maintain the preparation at a good condition. Each of the suboesophageal-prothoracic connectives was hooked up in the air by a recording electrode of $200\,\mu$ silver wire mounted on a micromanipulator. An indifferent electrode was provided by similar silver wire resting on wet cotton in the opened abdominal segments. In most cases the impulse discharges of the right and left auditory T large fibres (Suga & Katsuki, 1961) were displayed on the first and the second beams of an oscilloscope with three channels. A wave form of a sound stimulus or time signal was shown on the third beam alternatively according to need.

Several pharmacological agents in various concentrations (w/v of the physiological saline solution) were applied to the prothoracic ganglion. The change in the number of impulses in the T large fibres (post-synaptic fibres) was successively recorded on a running film after the application. The stimulating and the recording equipments used in the present work were the same as those described in the previous paper (Katsuki & Suga, 1960).

RESULTS

(1) *Excitors*

It was found that four drugs—picrotoxin, eserin, butyrylcholine and acetylcholine—brought about an increase of impulse discharges in the T large fibres. The effect of each drug, confirmed by several trials, will be described later. The T large fibre situated nearer to the sound source always discharged with more impulses than that of the opposite side. The difference in the intensity of a sound stimulating both tympanic organs was obviously recognized as the difference in the threshold and also in the number of impulses between a pair of T large fibres (Fig. 1 *a*).

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[*] $NaCl$, 12·16 g.; KCl, 0·23 g.; $CaCl_2(2H_2O)$, 0·27 g.; $Na_2HPO_4(12H_2O)$, 1·07 g.; $NaH_2PO_4(2H_2O)$, 0·05 g.; H_2O, 1000 g.

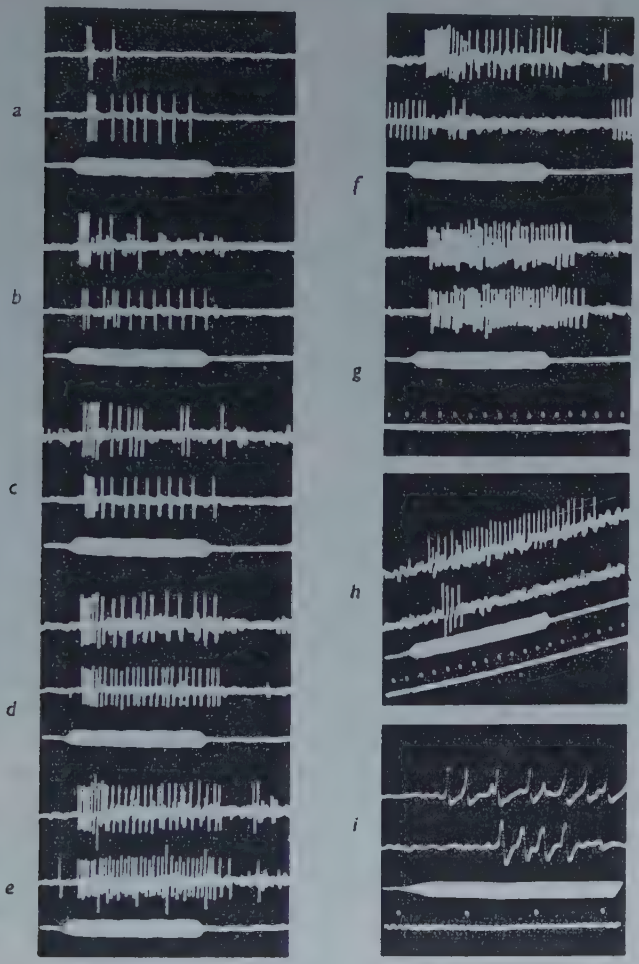

Fig. 1. Augmented response to a sound stimulus of both T large fibres after application of 0·1 % picrotoxin solution to the prothoracic ganglion (*b–g*) and contralaterally evoked impulses after cutting a tympanic nerve (*h* and *i*). (See text.)

(*a*) *Picrotoxin*

When 0·1% picrotoxin solution was applied to the prothoracic ganglion, impulse discharges of a pair of the T large fibres began to increase after about 6 min. from the application (*b*), and after 10 min. a train of impulses was observed not only on the left T large fibre, but also on the right as shown in Fig. 1 *d*. After 12 min. (*e*) after-discharges began to appear. The long-lasting train of impulses was suppressed by a tone burst following after about 18 min. (*f*). Such a suppression could be repeated for a few minutes after its appearance. In this concentration, the after-discharge became gradually shorter thereafter and the suppression became faint. After 26 min. the response pattern became stable (*g*). (The time signals are 10 msec.)

If one of the tympanic nerves was cut, many impulses of the ipsilateral T large fibre disappeared and a few impulses transmitted from the contralateral tympanic nerve always remained (*h*). The first impulse in the contralaterally evoked response delayed by about 8·0 msec. compared with that of the ipsilaterally evoked one (*i*). The time delay was 3·3 msec. in the shortest case. This result confirms the suggestion that the T large fibre is activated by some of the contralateral tympanic nerve fibres (Suga & Katsuki, 1961). When the material was washed with the physiological saline solution, the increased discharges began to decrease but the recovery was so slow that the number of impulses did not decrease to the original level even after 20 min. When 1 % picrotoxin solution was applied, many spontaneous discharges appeared in the T large fibres after 1 or 2 min. and then disappeared. The response to the tone burst also failed. But spontaneous discharges and the response reappeared after 5 or 6 min. Such a reappearance was always repeated twice or more. Finally a response with many impulses was found in some cases but not in other cases. With 0·01 % picrotoxin solution, the increase of impulse discharges was smaller compared with the case of 0·1 % picrotoxin.

(*b*) *Eserin*

Eserin applied to the prothoracic ganglion caused a remarkable increase of impulses in the T large fibre. The result shown in Fig. 2A was obtained by the application of 0·0001 % eserin solution to the prothoracic ganglion after increase of impulses in the right T large fibre by cutting the left tympanic nerve (*a* and *b* in Fig. 2A). Impulse discharges in the T large fibre contralateral to the cut tympanic nerve began to increase in response to a sound stimulus in about 4 min. after the application and then the ipsilateral T large fibre was also brought into excitation with one spike, which was delayed by 3–5 msec. as compared with the first impulse of the contralateral one (*c*). The number of impulses became almost constant after about 17 min. Two or three impulses evoked contralaterally were found on the T large fibre on the same side as the cut tympanic nerve (*d*). The time signal at the bottom of *d* represents 10 msec. Among the contralaterally evoked impulses the first impulse was delayed by 2·3 msec. as compared with the ipsilaterally evoked one in the shortest case (*e*). (The time signal is 1 msec.) When eserin solution more highly concentrated than 0·0001 %, for example 0·01 % eserin solution, was applied to the ganglion, after about 2 min. the impulses in the tonal response increased and also many spontaneous discharges were observed in the T large fibre. Both the T large fibres fired with trains of impulses after 3 min. and then ceased to fire completely. In contrast to picrotoxin, eserin did not cause any remarkable increase of impulses in the tonal response or any after-discharge.

This result may be well accounted for by the assumption that eserin is an anticholinesterase in the insect and that the activity of inhibitory neurons is also prolonged, so that the responses of the T large fibre are not augumented so prominently, whereas picrotoxin inhibits the inhibitory synapses between the T large fibre and inhibitory fibres, so that the T large fibre is fully activated by many tympanic neurons.

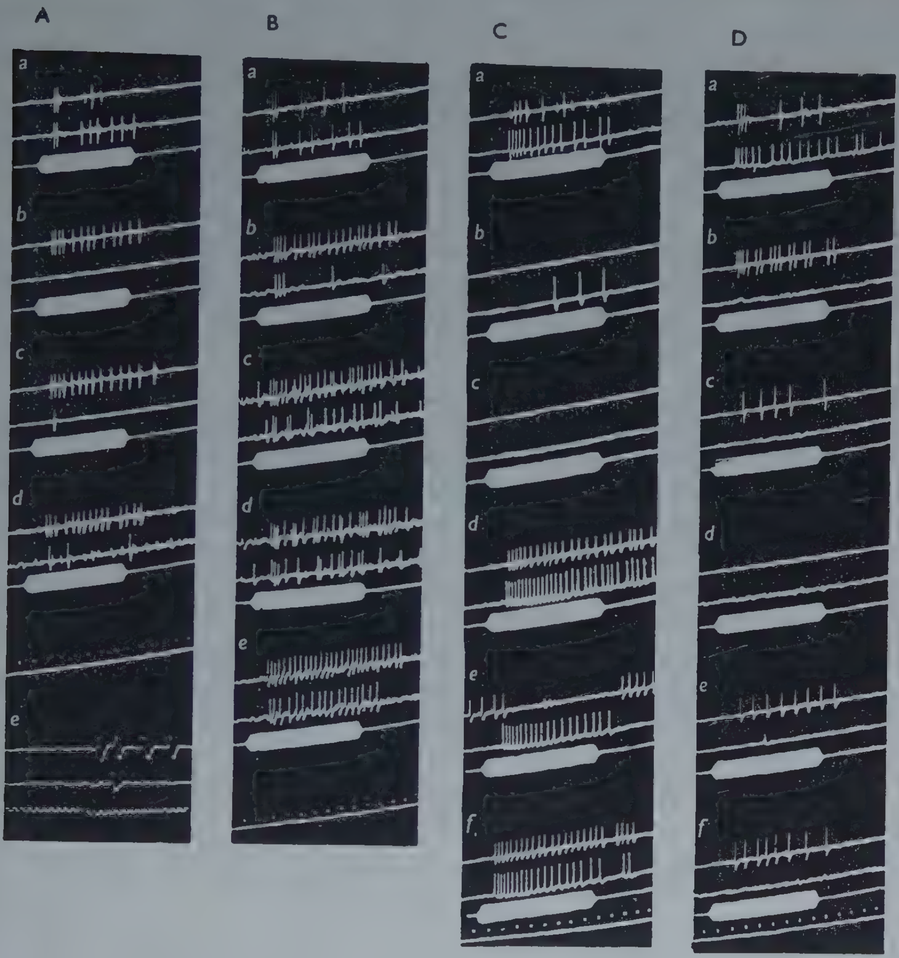

Fig. 2. A, Excitatory effect of 0·0001 % eserin solution applied to the prothoracic ganglion after elimination of the inhibitory effect from the contralateral tympanic nerve (*c*–*d*). Time delay between the impulses evoked ipsilaterally and contralaterally is 2·3 msec. (*e*). B, Augumented impulse discharges in the T large fibre by 1 % butyrylcholine solution (*b*–*e*). 1 % GABA (C) and 1 % γ-aminobutyrylcholine (D) solutions suppress impulse discharges in the T large fibres completely. (See text.)

(*c*) *Butyrylcholine*

1 % butyrylcholine solution was applied to the prothoracic ganglion connecting with both the tympanic nerves (*a* of column B in Fig. 2). When the number of impulses in the tonal response of the T large fibre was not very small, a conspicuous increase was observed after the application as shown in Fig. 2B. The impulse discharges in the response began to increase within 1 min. after the application (*b*), and spontaneous discharges were observed thereafter (*c* and *d*). The response became almost constant in 5 min. (*e*). No impulse evoked contralaterally by the activities of the tympanic nerves was found after cutting one of them. In many cases, however, when the number of impulses in a tonal response was very small, such an increase was not found after the application of 1 % butyrylcholine solution to the prothoracic ganglion

with both tympanic nerves intact. When the solution was applied to the prothoracic ganglion after cutting one of the tympanic nerves, impulses in a tonal response in the contralateral T large fibre always increased, but the T large fibre on the cut side did not always fire. Such was not the case following the application of picrotoxin solution.

Those results can be explained as follows. The effect of butyrylcholine is influenced by the activity of the inhibitory synapse, namely, when the inhibitory effect from the opposite side is strong, the number of impulses in the T large fibre becomes less and the excitatory effect of butyrylcholine does not appear distinctly except when the solution is applied after cutting the contralateral tympanic nerve, because the inhibitory effect on the T large fibre from the tympanic nerve is eliminated by cutting it.

(*d*) *Acetylcholine*

The effect of acetylcholine was also excitatory even though it seemed to be slightly less effective than butyrylcholine. When 1 % acetylcholine solution was applied to the prothoracic ganglion after cutting one of the tympanic nerves, the number of impulse discharges in the contralateral T large fibre began to increase within 0·5 min. after the application. In 15 min. the contralateral impulses further increased and the ipsilateral T large fibre discharged an impulse. Several spontaneous discharges also appeared. But the number of impulses in the tonal response of the large fibre did not increase as with picrotoxin. The time delay between the impulses evoked contralaterally and ipsilaterally was 3·2 msec. in the shortest case. Washing with the physiological saline solution made the number of impulses decrease and restored the original response after 2 or 3 min.

(2) *Inhibitors*

Four drugs, GABA, γ-aminobutyrylcholine, D-tubocurarine and strychnine acted as inhibitors of electrical activity in the T large fibre.

(*a*) *GABA*

1 % GABA was applied to the prothoracic ganglion (Fig. 2C, *a*). Impulse discharges in the T large fibres began to decrease after 1·5 min., and after 5 min. they disappeared completely (*c*). Washing the prothoracic ganglion with the physiological saline solution brought back the tonal response of the T large fibre. In many cases, within 1 min. after washing, the T large fibre discharged many impulses even after the cessation of a sound stimulus (*d*). Such after-discharges were often suppressed by tone bursts (*e*). But a train of after-discharges became shorter and shorter afterwards (*f*) and the original responses finally returned on the T large fibres. Reversibility was also complete when the response of the T large fibre to the tone burst was suppressed by the application of 10 % GABA solution. 0·1 % GABA solution had almost no effect on the response of the T large fibre. Even if the number of impulses in a response slightly decreased at first, it soon returned to the original level.

(*b*) *γ-Aminobutyrylcholine*

1 % γ-aminobutyrylcholine suppressed the electrical activity of the T large fibre in the same way as GABA did and the reversibility of the effect was also complete (Fig. 2D). γ-Aminobutyrylcholine solution was applied after the increase of the number of

impulses on the right T large fibre consequent upon cutting the left tympanic nerve (*a* and *b*). The enhanced response by elimination of the inhibitory effect on the T large fibre from the left tympanic nerve consisted of only five impulses within 0·5 min. after the application (*c*). After 5 min. the response disappeared entirely (*d*). However, it returned after 3 min. washing with the physiological saline solution (*e* and *f*). The reversibility was the same even if 10% γ-aminobutyrylcholine solution was applied to the prothoracic ganglion.

(*c*) *d-Tubocurarine chloride pentahydrate*

If the prothoracic ganglion was treated with 0·03% D-tubocurarine solution, impulse discharges in the T large fibre began to decrease within 3 min. after the application and all the tonal responses disappeared thereafter. 0·03% D-tubocurarine solution suppressed the increased discharges evoked by the application of 1% acetylcholine solution, but did not cause them to disappear. But D-tubocurarine solution in higher concentration (0·3%) caused them to disappear completely. The vanished impulses reappeared on the T large fibre after washing of the ganglion with the physiological saline solution.

(*d*) *Strychnine*

When the prothoracic ganglion was treated with 1% strychnine solution, impulse discharges in the T large fibre decreased after about 1 min. and eventually disappeared, as after treatment with metrazol. After washing the prothoracic ganglion with the physiological saline solution the response to sound stimulation returned in the T large fibre within 9 min. after washing.

(3) *Response frequency range and number of impulses after the treatment with picrotoxin*

After the application of picrotoxin solution to the prothoracic ganglion the threshold of the tonal response of the T large fibre became higher and the latency became longer in spite of the remarkable increase of impulse discharges.

In the normal condition a large difference in the threshold between the two T large fibres was found for various sounds in the frequency range of the response as reported previously (Suga & Katsuki, 1961). However, it was noted that the difference became small after the application of picrotoxin solution to the prothoracic ganglion. One of the experiments is shown in Fig. 3 as an example. The response ranges of the T large fibres were measured before and after the application of 1% picrotoxin solution. The threshold of the left T large fibre (near to a sound source) became higher (○---○) than the original one (○—○), but that of the right T large fibre (far from the source) became lower (●---●) than the original one (●—●). As that result, the difference in the threshold between the two fibres became small.

On the other hand, the increased information carried by the two T large fibres by reason of the central inhibitory interaction (Suga & Katsuki, 1961) was disturbed by the suppression of interaction following the application of picrotoxin solution. That is, the ratio between the number of impulses sent through one of the T large fibres and the number sent through the other became small (compare *a* and *g* in Fig. 1) and the number of impulses in both changed at the same rate with change of sound intensity. No increase in the number of impulses was found after cutting one of the tympanic nerves. These facts will be interpreted on the assumption that the T large fibres are fully activated by the ipsilateral and contralateral tympanic nerves after the suppression of the activity of inhibitory synapses by picrotoxin.

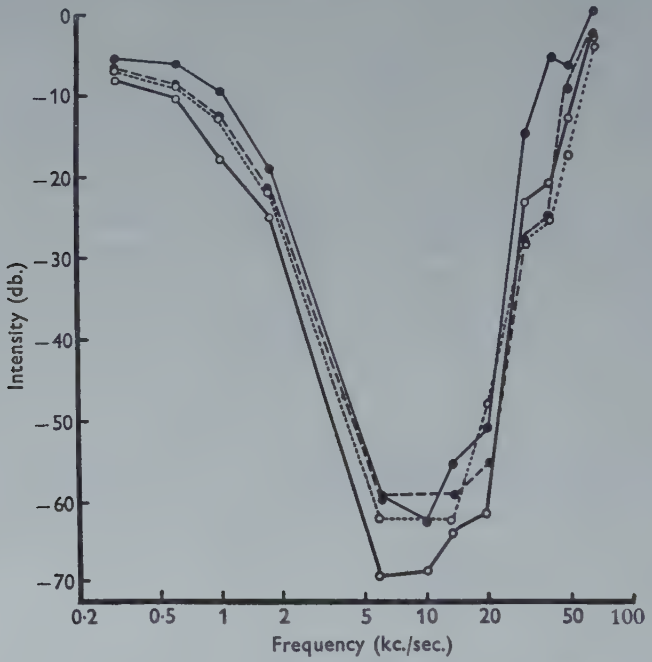

Fig. 3. Change in threshold after application of 1 % picrotoxin solution. The ordinate and abscissa represent the intensity and frequency of a sound respectively. Threshold curve of T large fibre near to the sound source (○—○) and that far from the source (●—●) before the application. Symbols ○---○ and ●---● represent the threshold curves after the application respectively. (See text.)

(4) *Electrical stimulation*

The left tympanic nerve was cut and its proximal end was hooked up in the air with a pair of silver wire electrodes. Long or short electric square pulses were given to the cut end of the tympanic nerve through these electrodes during application of a sound stimulus. The sound stimulus excited the right tympanic organ and the information was sent to the brain as a train of impulses through the right T large fibre only. The short square pulses (of a few milliseconds duration) applied to the left tympanic nerve gave rise to silent periods in a train of impulses on the right T large fibre, while the left T large fibre fired with one impulse for each square pulse. A negative square pulse of 100 msec. duration was applied to the left tympanic nerve at the same time as the sound stimulus. Almost all impulses in the right T large fibre were suppressed. On the other hand, the left T large fibre discharged an impulse at the onset of the square pulse and never discharged repetitively. However, if the right tympanic nerve was cut, the left T large fibre discharged with four or five impulses at the onset of the negative square pulse.

A positive square pulse of 100 msec. duration had no effect on the response to the tone burst on the right T large fibre even if it was applied to the left tympanic nerve at the same time as the tone burst. The left T large fibre discharged one impulse only, at the end of the square pulse. These experimental results described above further confirm the existence of the proposed innervation modus of the T large fibre by the tympanic nerve.

DISCUSSION

(1) *Functional connexion between the tympanic nerve and the T large fibre*

The effects of the excitatory agents are explained as follows. Picrotoxin, known as an inhibitor of the inhibitory synapses in a crustacean stretch-receptor, always brought about a remarkable increase in the number of impulses in the T large fibres. This fact accounted for the similar suppression of the inhibitory synapses between the auditory T large fibres and inhibitory interneurons. In contrast to picrotoxin, the agents eserin, butyrylcholine, and acetylcholine produced an excitatory effect on the synapses between the tympanic nerve and the T large fibre, and between the tympanic nerve and the inhibitory interneuron. The results showed that the change in the number of impulses after application of the pharmacological agents seemed to be always dominated by the activity of the inhibitory fibre in each specimen, that is, the increase of impulses was not so clear when the activity of the inhibitory fibre was high, but it was remarkable when it was low. However, when the agent was applied after cutting one of the tympanic nerves, i.e. after the elimination of the inhibitory action on the T large fibre from the opposite side, the T large fibre on the side opposite to the cut always discharged many more impulses, but the T large fibre on the same side did not.

Further, GABA (known as an excitor of the inhibitory synapses in Crustacea) strongly suppressed impulse discharges in the T large fibre, which may be due to the activation of the inhibitory synapse, though it is a fact that in the lobster GABA blocks the thoracic receptor which has no inhibitory synapses (Kuffler & Edwards, 1958). γ-Aminobutyrylcholine also extinguished impulse discharges on the T large fibres as GABA did.

Thus the pharmacological experiments have afforded further evidence of the central interaction between the tympanic nerves and the T large fibre which was originally discovered by the electrophysiological study of the central auditory mechanism, and have confirmed that the T large fibre has excitatory synapses with the pair of tympanic nerves and inhibitory synapses with the inhibitory interneurons activated by the tympanic neurons.

An anatomical study of these nerve nets has been made, but no definite configuration has so far been discerned. The time delay between the first impulse contralaterally evoked in the T large fibre and that ipsilaterally evoked must be due to the longer path from the contralateral tympanic nerve fibres to the T large fibre. The shortest time delay was found after the application of eserin solution to the prothoracic ganglion, being 2·3 msec. The inhibitory effect on the T large fibre from the contralateral tympanic nerve fibres appeared with the time delay of 4·6 msec. (Suga & Katsuki, 1961). Fig. 4 shows schematically the functional connexions between the tympanic nerves and two T large fibres.

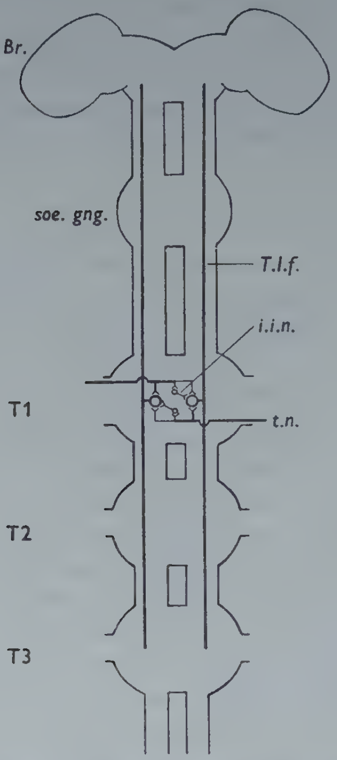

Fig. 4. Schematic representation of the tympanic system revealed by electrophysiological and pharmacological studies. *Br.*, brain; *i.i.n.*, inhibitory interneuron; *soe. gng.*, suboesophageal ganglion; T 1, 2 and 3, pro-, meso-, and metathoracic ganglia; *T.l.f.*, T large fibre; *t.n.*, tympanic nerve.

(2) *Synaptic transmission*

From the fact that the pharmacological agents described above, known to act on the synapses of Mammalia and Crustacea, gave rise to the change in impulse discharges of the T large fibre, it may be concluded that synaptic transmission between the tympanic neurons and the T large fibre and between both neurons and the inhibitory interneurons is carried out through some chemical processes. The concentrations of the agents used in the present work were higher than those reported by other authors as applied to such isolated materials. If the agents were applied after opening the sheath of the prothoracic ganglion, events similar to those described above were caused by the solutions of lower (by almost 10%) concentration in all cases. As it was found that the inhibitory effect on the T large fibre was weakened by peeling off the sheath of the prothoracic ganglion and impulse discharges on the T large fibre increased, no attempt was made to discover the minimum effective concentration of the test solution, because the condition of the material was unstable.

It is well known that picrotoxin and GABA are competitive antagonists at the excitatory synapses of the cat brain (Purpura *et al.* 1957) and at the inhibitory synapses of crustacean stretch-receptors (Elliott & Florey, 1956; Edwards & Kuffler, 1957) and muscle (Grundfest *et al.* 1959; Reuben *et al.* 1959), so it is highly probable that these agents act antagonistically on the synapses in the prothoracic ganglion as on those of

Crustacea and that an antagonism might be also found between acetylcholine and D-tubocurarine. Acetylcholine and D-tubocurarine which can cause certain effects on the cat brain have been revealed to be without effect on lobster neuromuscular synapses, although acetylcholine acted as an excitor on the insect synapses. Both strychnine and metrazol suppress the activity of the T large fibre. However, the response of the T large fibre to a sound stimulus returned after washing the prothoracic ganglion with the physiological saline solution. Therefore it seems that these two drugs act inhibitorily on the insect synapse. These results suggest that synaptic transmission in the prothoracic ganglion may be carried out by a mechanism different from that in mammalian synapse. It should be possible to reveal more details of the mechanism by intracellular potential recording, but this has been unsuccessful as yet.

SUMMARY

1. Picrotoxin, eserin, butyrylcholine and acetylcholine bring about increase of impulse discharges on the T large fibre in the cord. Picrotoxin gives conspicuous increase of impulse discharges in the response of the T large fibres to sound, while the excitatory effects of the latter three agents are not so conspicuous.

2. Such effects can be explained on the assumption that picrotoxin inhibits the inhibitory synapses, so that the T large fibre is fully activated by both tympanic nerves, while the latter three agents activate both the excitatory and inhibitory fibres.

3. GABA, γ-aminobutyrylcholine and D-tubocurarine act reversibly as inhibitors of the activity of the T large fibre. The response evoked in the T large fibre may be suppressed by the activities of the inhibitory interneurons activated by the tympanic nerve fibres.

4. After the application of picrotoxin solution to the prothoracic ganglion the threshold of the T large fibre near to a sound source rises while that of the opposite side falls. The inhibitory effect seems to be eliminated by the drug action and the T large fibre is activated by both the ipsilateral and the contralateral excitatory fibres.

5. The increased information about a source of sound which arises from the central inhibitory interaction is disturbed by the application of picrotoxin.

6. The conclusion that the T large fibre has excitatory synapses with the tympanic nerves, and inhibitory synapses with the inhibitory interneurons activated by the tympanic neurons, has been confirmed pharmacologically.

7. The inhibitory interneurons are activated not only by the natural activity of the tympanic nerve, but also by activity elicited electrically with square pulses.

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THE PHYSIOLOGY OF BLOOD TREHALOSE AND ITS FUNCTION DURING FLIGHT IN THE BLOWFLY

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INTRODUCTION

Insects of the order Diptera possess flight muscles capable of contracting at very rapid rates for long periods. The energy for this activity is derived mainly, if not exclusively, from the aerobic oxidation of carbohydrate (Chadwick & Gilmour, 1940; Davis & Fraenkel, 1940; Williams, Barness & Sawyer, 1943; Chadwick, 1947; Wigglesworth, 1949; Hocking, 1953; Clements, 1955; Hudson, 1958). Glycogen has often been implicated as the reserve substrate for flight, mainly on the basis that this substance appeared to be the only carbohydrate present in appreciable amounts and that it decreased in amount as a function of flight duration. However, Wyatt & Kalf (1956, 1957) more recently showed that the non-reducing disaccharide, trehalose, is found in large concentrations in the haemolymph of several insects, and two other studies independently demonstrated trehalose to be an insect blood sugar (Howden & Kilby, 1956; Evans & Dethier, 1957). Since that time the list of insects having trehalose as a blood sugar has been extended, and several studies have been made of the enzymes and pathways involved in the metabolism of this sugar (cf. Wyatt, 1961; Sacktor, 1961). Although several suggestions have been made, there is little experimental evidence on the physiological role of this disaccharide in insects. Since practically all of the studies concerning the flight substrate in Diptera were carried out prior to the discovery of trehalose, the main purpose of our work was to examine the relationship between the two parameters, flight and trehalose, in the blowfly. A preliminary account has been published elsewhere (Clegg & Evans, 1961).

MATERIALS AND METHODS

Phormia regina Meigen, the blowfly used in this study, was reared by the method of Hill, Bell & Chadwick (1947). Only males were used to avoid complications arising from sex differences.

Analytical methods

Whole blood was collected from adult flies by the method of Evans & Dethier (1957). These workers found that the very small number of formed elements in adult blood do not contain an appreciable amount of carbohydrate; accordingly, whole blood was used in this study.

The identification and separation of carbohydrates was carried out by paper chromatography (Evans & Dethier, 1957). For quantification the carbohydrates were eluted from the paper with water into small beakers. These eluates were then frozen on dry-ice, and taken to dryness *in vacuo* over $CaCl_2$. A known volume of water was added, and an appropriate aliquot taken for colorimetric assay of carbohydrate (Evans & Dethier, 1957) or radioactivity determinations. When entire chromatograms were assayed, the glucose and trehalose areas were cut out for elution and the remainder of the paper was eluted serially in 2 cm. strips.

Glucose-U-^{14}C was obtained from Nuclear-Chicago; mannose-U-^{14}C, galactose-1-^{14}C, fructose-U-^{14}C, and inulin-carboxyl-^{14}C were obtained from 'Calbiochem', Los Angeles, California. All of these were demonstrated to be chromatographically pure before use. Radioactive samples were plated on stainless steel planchettes and assayed with a thin-window Geiger-Müller tube in an automatic counter. Corrections for self-absorption were made when necessary, most samples being counted at infinite thinness. All counting errors are given at the 1 % level. Isotopically labelled trehalose, not available commercially, was synthesized by incubating intact fat body of the roach, *Leucophaea maderae*, with glucose-U-^{14}C of high specific activity. This tissue converts exogenous glucose into trehalose and liberates it into the medium with high efficiency (specific activity of the trehalose synthesized was 40–60 % of the specific activity of the precursor glucose). Isolation and purification of ^{14}C-labelled trehalose from the incubation medium was accomplished by the above-mentioned chromatographic procedures.

Bodenstein's (1946) saline, buffered to pH 7 with 0·01 M phosphate, was used for tissue incubations and as a carrier for injected sugar solutions.

Injections of sugar solutions were made with a micrometer-driven microinjection apparatus, containing a 50 μl. syringe equipped with a 27-gauge needle. To insure minimum damage with such injections, a very fine-tipped glass needle (made on a micro-electrode puller) was glued over the metal needle. Injections were made into the scutellum after the fly had been mounted on a clay support. The volume and error of delivery of this apparatus was estimated by determining the amount of glucose delivered by unit turn of the micrometer after the syringe had been filled with a known concentration of glucose. In every case, 0·524 μl. (± 0·008 S.E.) was the volume injected.

Tissue preparations

The half-thorax preparation was made by anaesthetizing the fly with carbon dioxide (Williams, 1946), severing the abdomen at its junction with the thorax, and then carefully pulling off the head with attached mid-gut. After removal of the wings and legs, this isolated thorax was hemisected along the mid-line with a thin razor blade. The thoracic ganglion and salivary glands were removed under a microscope. If the dissection is done carefully, the flight muscle remains intact, and comprises the bulk of the tissue in such 'thorax-halves', although a few fat body cells are also present. These preparations were rinsed in saline three times, gently blotted after each rinse, and then placed in the incubation flask. For weight determinations the muscle mass was dissected from the thorax, blotted gently, and placed on a small pre-weighed piece of aluminum foil. For determination of dry weight, the muscle was placed in an oven at 100° C. for 60 min., at which time water loss was complete. All weighings were carried out with a Roller-Smith balance (sensitive to 0·05 mg.).

Preparations of intact fat body were obtained from the abdomens of male flies which had been reared on 2 M. glucose from eclosion. The abdomen was severed at the thoracic junction, and the most posterior abdominal segment was carefully removed under saline. The abdominal viscera were removed and the larger pieces of fat body teased out with glass needles and transferred through three saline washes with small glass pipettes. The pipettes were treated with silicone to prevent the fat body from sticking to the glass. The abdomens of three to five flies usually contained enough fat body for a single incubation (about 1·5 mg. dry weight). This 'pooled' fat body was then incubated in 0·1 ml. of the appropriate medium. Tissue dry weight was determined in the same manner as for flight muscle. Incubations were carried out at room temperature (25–27° C.) with gentle shaking.

Flight procedure

Males, 4–5 days after eclosion, were used for all flight studies. They were maintained on a 2M glucose solution sprayed twice daily on filter paper. Such flies can be considered replete with respect to carbohydrate stores. The flies were mounted for flight, using a method similar to the one Chadwick & Gilmour (1940) developed for *Drosophila*. This consisted of suspending the fly from a thin cardboard strip attached at one end to the dorsal abdomen with a small drop of glue, and at the other end to the substratum. The fly was now suspended in a fixed position. The tarsi were removed to promote flight (Friedman, 1959). Continuous flight could be initiated in about 60% of such flies by brushing the ventral surface of the body. Flight was often spontaneous under these conditions. Measurements of the wing-beat frequency were made with a 'Strobotac' (type 631-B, General Radio Company) by placing the emission tube 6 in. from the suspended fly. Use of the strobotac on 'flying' insects has been outlined by Chadwick (1939), Williams & Galambos (1950), and Williams & Chadwick (1943). All flight experiments were carried out under relatively constant conditions of light intensity, humidity and temperature.

RESULTS AND DISCUSSION

Blood sugars and the flight process

Blood carbohydrates as a function of flight duration. To our knowledge the only previous indication that blood trehalose is utilized by the flight muscles of Diptera during flight was the decrease in blood trehalose concentration observed during continuous flight of the blowfly, *Phormia regina* (Evans & Dethier, 1957). This result has been confirmed in the present study by comparing the blood sugar concentrations of two groups of similar flies, one flown for 3 hr. and the other rested for the same period. An aliquot of blood was then removed and assayed quantitatively as described. The results (Table 1) showed that flight reduced the blood trehalose concentration to about 1/5 of the control value while glucose concentration fell much less sharply, The only other blood carbohydrate fraction, listed as base line carbohydrate ($R_F = 0$), was not altered appreciably by flight. This fraction represents any carbohydrates that do not migrate in these particular solvent systems; while it is likely that glycogen or a similar polysaccharide constitutes the bulk of this fraction (Evans & Dethier, 1957), sugar phosphates might also be present.

The time course of wing-beat frequency during flight to exhaustion. Next, the relationship of blood trehalose to the rate of energy expenditure during flight was sought.

Fortunately, it has been shown that the wing-beat frequency (WBF) is a rather good index of the rate of oxygen consumption throughout flight to exhaustion in three species of *Drosophila* (Chadwick & Gilmour, 1940; Chadwick, 1947). Since flight energy is derived largely, if not solely, from aerobic pathways in the Diptera, the WBF is also a valid measure of the rate of energy expenditure.

Table 1. *The effect of flight on blood carbohydrates*

Base-line carbohydrate represents non-migrating carbohydrate ($R_F = 0$).

		Average blood concentration (g./l.)		
Treatment	No. of flies	Glucose	Trehalose	Base-line carbohydrate
Rested for 3 hr.	13	1·88	27·10	0·54
Flown for 3 hr.	15	1·03	4·59	0·51
	Rested/flown	1·8	5·9	1·1

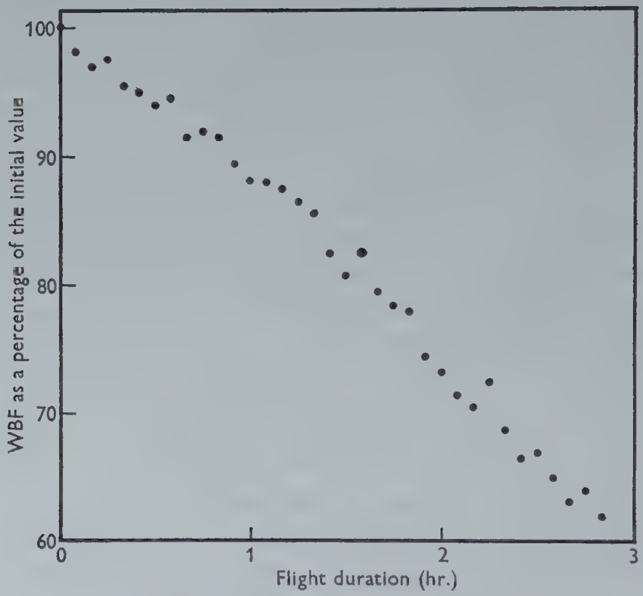

Fig. 1. Wing-beat frequency (WBF) as a function of the flight duration of flies maintained from eclosion on 2 M glucose.

We first measured the time course of WBF during flight to exhaustion. The flies were selected and mounted for flight as described. WBF measurements were made every 5 min. and the values then were expressed as a percentage of the initial rate to compensate for individual differences. Individuals having a flight duration of less than 2 hr. were discarded. This was done to standardize the population of flies used since it has been shown that flight *duration* is a function of the nutritional state of the fly (Williams *et al.* 1943). The results of these experiments are summarized in Fig. 1 Although individual flies showed some deviation the trend was the same, much like Chadwick (1939) found for *Drosophila* repleta, and Smyth (1952) for *Phormia regina* For *Phormia*, WBF falls gradually and continuously during flight. Eventually, a WBF is reached below which flight does not occur and the animal may be termed 'exhausted'. The average initial WBF was about 11,400 cyc./min. and the 'exhausted' WBF about 7000 cyc./min.

Blood glucose and trehalose as a function of wing-beat frequency. Since substrate depletion has been implicated as a cause of flight exhaustion (Chadwick & Gilmour, 1940; Williams *et al.* 1943; Wigglesworth, 1949), experiments were carried out to determine what relationships, if any, existed between blood sugar concentrations and the WBF.

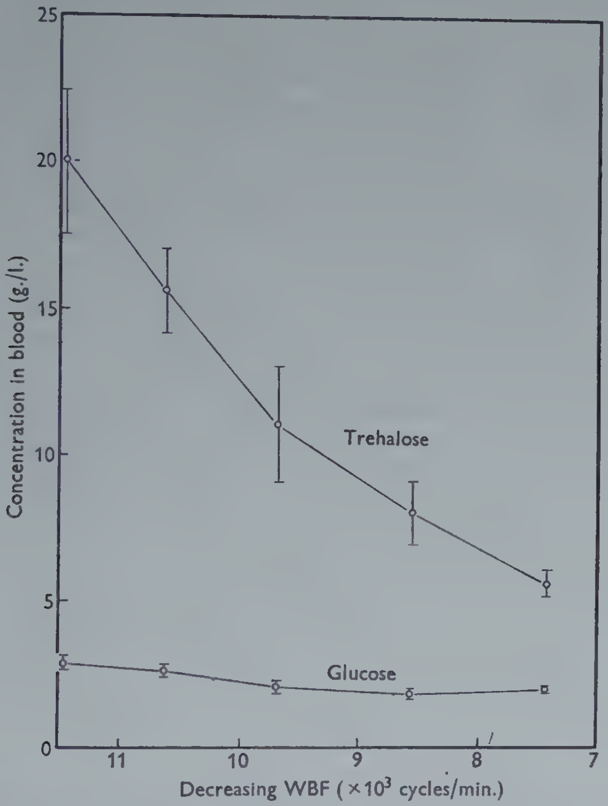

Fig. 2. Relationship between the wing-beat frequency (WBF) and the concentrations of blood glucose and trehalose. The data were grouped by averaging WBF observations made over intervals of 1000 cyc./min. from 12,000 to 7000 cyc./min. The bars represent the standard errors for the sugar concentrations; each point represents the averaged results from at least ten individuals.

Flies were flown until the desired WBF was reached and an aliquot of the blood immediately removed and assayed quantitatively for glucose and trehalose. Determinations were made separately on each fly so that the results (Fig. 2) could be analysed statistically. Blood trehalose concentration fell with WBF over the entire range of WBF, while blood glucose did not change significantly. The variability in trehalose concentration was large but the downward trend is clear. The smaller variability seen in trehalose concentration as the point of exhaustion is reached is noteworthy. Clearly, WBF and the concentration of trehalose in the blood are correlated.

Wing-beat frequency as a function of blood glucose and trehalose administered by injection. Since others have shown that the administration of carbohydrate by feeding (Wigglesworth, 1949; *Drosophila*) or injection (Hudson, 1958; *Phormia*) restores flight in exhausted flies, there are strong indications that exhaustion results from an insufficient rate of supply of substrate to the flight muscles. Other less direct evidence supports this view (Chadwick, 1947; Williams *et al.* 1943). Accordingly, the relation between blood trehalose and the WBF might be more than a correlation: trehalose concentration might determine the WBF. A direct test of this hypothesis was performed.

Flies were flow to exhaustion and the WBF at which they stopped was designated as the exhausted WBF. Each fly was then injected serially with 52, 105 and 210 μg. of trehalose or glucose dissolved in saline. In all cases 0·524 μl. was the volume injected. After the first injection each fly was rested for a 3 min. equilibration period and then flown. The WBF was recorded every 10 sec. for the first minute of flight; then these values were averaged and designated as the WBF after injection. The flies were then exhausted before the next injection, and the process repeated. Following the last injection, each fly was fed 2 M. glucose to repletion, rested for 30 min., and again flown. The WBF thus obtained was designated as the WBF after feeding. This value may be considered as the maximum for a given fly. A total of eight flies was used for the trehalose injections and five flies for the glucose injections.

If WBF were determined by the concentration of blood trehalose, then an increase in the concentration of this sugar in the blood of an exhausted fly should restore continuous flight. Moreover, the increase in trehalose concentration should be correlated with the increase in the WBF measured during subsequent flight. Table 2 summarizes the results of these experiments in which both of the predictions were confirmed. The largest amount of trehalose injected (210 μg.) restored the WBF to its maximum value. Since the blood volume of *Phormia* is about 7 μl. (Table 4), the injection of 210 μg. of trehalose should increase the concentration in blood to about 30 g./l., which is roughly the maximal concentration found in the blood of *Phormia* (Table 1, and Evans & Dethier, 1957). We, therefore, conclude that, under these conditions, blood trehalose concentration determines the WBF.

Glucose injections produced a similar effect in the few cases studied (Table 2), emphasizing the fact that this substance must not be overlooked as an important blood sugar (Evans & Dethier, 1957). However, at the concentrations of glucose and

Table 2. *Wing-beat frequency (WBF) of exhausted flies following injections of glucose or trehalose into the blood*

	Trehalose injections		Glucose injections	
Treatment	Mean WBF ± S.E. (× 10³ cyc./min.)	As a percentage of the 'Fed' WBF	Mean WBF (× 10³ cyc./min.)	As a percentage of the 'Fed' WBF
Exhausted	7·73 ± 0·16	69	8·44	74
Injected:				
52 μg.	8·91 ± 0·18	80	9·72	85
105 μg.	10·13 ± 0·18	91	10·56	93
210 μg.	11·08 ± 0·19	99	11·25	99
'Fed'	11·18 ± 0·20	100	11·34	100

trehalose normally present in the blood (Table 1, Fig. 2), trehalose will be, quantitatively, the more important metabolite. That desiccation was not the limiting factor was indicated by the observations that exhausted flies either refused to drink water or did not resume flight if any was imbibed, as was also the case with *Drosophila melanogaster* (Wigglesworth, 1949). When the carrier saline was injected into exhausted flies, flight ability was never restored (eight cases). It should be pointed out that experiments of this type must be carried out with great care. Several flies were in some manner injured by the repeated injections, refusing to fly after the injection had been made. In some cases the fly responded to initial injections but not to subsequent ones. Such flies were fed on 2M. glucose, rested, and stimulated to fly. In no case was continuous flight observed, indicating that these were not exceptions to the pattern seen in Table 2, but rather were unable to fly due to some sort of mechanical trauma associated with the injections.

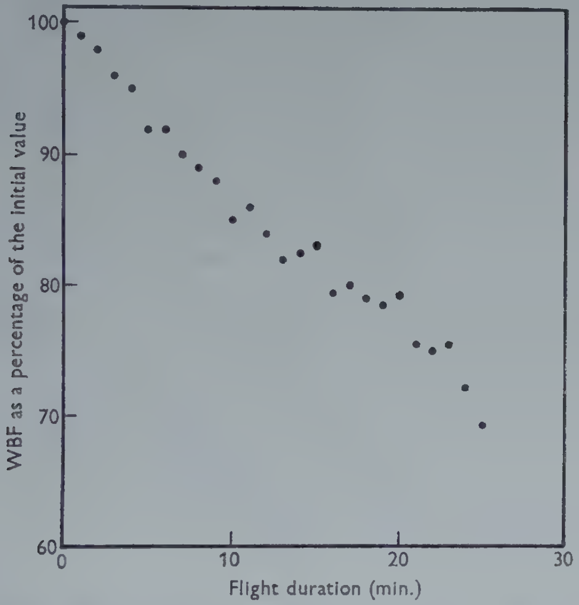

Fig. 3. Wing-beat frequency (WBF) as a function of the flight duration of exhausted flies following the injection of 210 μg. of trehalose.

Wing-beat frequency of exhausted flies following the injection of trehalose. The curve shown in Fig. 3 represents data from ten flies which had been exhausted, injected with 210 μg. of trehalose, and then reflown. The WBF was recorded every minute until exhaustion was reached. Although the duration of flight was obviously much shorter, the general shape of this curve is very similar to the one constructed from 'normal' flies (Fig. 1), indicating that similar factors were operating in the two groups of flies. Hudson (1958) estimated that *Phormia* utilized carbohydrate at a rate of about 15 μg./min./fly during flight. The introduction of 210 μg. of trehalose should, therefore, maintain flight for about 14 min. The average flight duration of this group of flies was just under 18 min., a figure that is in very good agreement with the calculated one.

Proposed relationship between blood trehalose and flight metabolism. On the basis of the foregoing observations it is proposed that the concentration of blood trehalose is a rate-limiting physiological factor determining the WBF and, therefore, the rate at which energy is expended by the flight muscles of *Phormia regina* during continuous flight. During prolonged flight blood trehalose continually falls, which obviously means that its utilization slightly exceeds its supply. As a consequence WBF declines, reflecting the declining blood trehalose concentration. Exhaustion would result when the concentration of this blood sugar reached some threshold concentration, below which trehalose could not be supplied to the flight muscles at a rate sufficient to permit continuous flight. This complements the studies of Wigglesworth (1949) and Williams *et al.* (1943), from which it was concluded that flight exhaustion was reached when glycogen could no longer be mobilized fast enough to meet the demands of active flight muscle. More will be said about this later. However, it must be pointed out now that the proposed relation between blood trehalose and the WBF need not necessarily apply during very brief flights when the fly is replete with carbohydrate. The role played by flight muscle glycogen (in terms of WBF regulation) under these conditions will be difficult to resolve since there appears to be no way to produce sudden alterations in flight muscle glycogen and then measure the effect on the WBF.

Flight muscle in vitro

Even before the discovery by Wyatt & Kalf (1956) that trehalose was an insect blood sugar, Sacktor (1955) had demonstrated that homogenates of housefly thoraces could metabolize this sugar at rates similar to those for glycogen and glucose. Thus, flight muscle possessed the metabolic pathways necessary for the oxidation of trehalose. However, since the structural integrity of the muscle was destroyed, Sacktor's study does not necessarily show that trehalose can be removed from the environment by intact muscles. The mechanism of this uptake is of some interest since metazoan cells are, in general, not very permeable to disaccharides, at least not in terms of the rates involved here. A respiratory study of intact flight muscle *in vitro* was undertaken in hopes of gaining information on this problem.

Conventional Warburg studies were carried out on half-thorax preparations. The

Table 3. *Respiration of intact flight muscle* in vitro

The main compartment of the flask contained 2·0 ml. of Bodenstein's saline buffered to pH 7 with 0·01 M phosphate. Glucose and trehalose, when present, were at a final concentration of 30 mM. The centre well contained 0·2 ml. of a 10% KOH solution. After each flask was prepared, six half-thorax preparations were placed in the main compartment, the flask was mounted on the manometer and immediately placed in the water bath for an equilibration period of 5 min. Readings were taken every 5 min. for at least 1 hr. The temperature of the water bath was 28° C., and the gas phase was air. The column marked 'Treatment' refers to the state of the flies before the muscle preparations were made.

Treatment	Substrate added (final conc.)	No. of experiments	Q_{O_2} ± S.E. (mm.^{3}/mg. dry wt. muscle/hr.)
Fed normally	None	12	11·5 ± 0·6
	30 mM. glucose	8	11·7 ± 0·8
Flown to exhaustion	None	13	4·9 ± 0·5
	30 mM. glucose	9	11·0 ± 0·5
	30 mM. trehalose	9	10·8 ± 0·5

conditions and results of these studies are summarized in Table 3. The endogenous respiration of preparations from normally fed flies was very high and could not be elevated by addition of substrate to the flask. However, if the flies were flown to exhaustion before the preparations were made, the endogenous rate was decreased. This rate could then be elevated to a level near that of muscle from fed flies by the addition of either 30 mM. glucose or trehalose. These results on intact muscle are essentially the same as those which Sacktor (1955) obtained using homogenates, and show that intact flight muscle can remove and metabolize exogenous trehalose. The R.Q. of preparations from normally fed flies was found to be 1·09 by the 'indirect-method' of Warburg (Umbreit, Burris & Stauffer, 1957), and respiration was linear with time for more than 1 hr. Sacktor (1955) suggested that glycogen stored in the flight muscle was the endogenous respiratory substrate since he could show a correlation between endogenous respiration and glycogen content of the thorax. If trehalose were also stored in the flight muscle it might contribute to this endogenous rate. We therefore analysed the flight muscle for trehalose. Muscle was dissected as quickly as possible from the thorax of well-fed flies, washed in saline, blotted dry, and frozen on dry ice until enough was collected for the determination (about twenty flies were used). The muscle was then weighed quickly, homogenized in 80% methanol, and centrifuged. The supernatant was decanted and the pellet washed twice with methanol. The combined supernatant was taken to dryness under vacuum, dissolved in a small amount of water, and chromatographed *in toto*. After development of the chromatogram, the trehalose was eluted and assayed colorimetrically. Another batch of muscle, removed from similar flies, was used to determine the amount of 'cell water' per unit wet weight of muscle. The trehalose content amounted to only 0·25 μg./mg. wet weight muscle or 0·31 μg./μl. cell water. Thus, it is unlikely that trehalose contributes appreciably to the endogenous respiration of these isolated muscles. These results also indicated that a steep concentration gradient exists for trehalose between the blood (10–30 g./l.) and the flight muscle (about 0·3 g./l.). The pellet from the methanol extraction was analysed for glycogen following the procedure of Hudson (1958). Glycogen was found at a concentration of about 28 μg./mg. wet weight muscle (as glucose); therefore, it is present in adequate amounts to subserve the endogenous respiration of these thorax halves. The reduction in the endogenous respiratory rate produced by flying the flies to exhaustion (Table 3) very likely reflects diminution of this thoracic glycogen.

Further studies were now undertaken to determine the rate at which trehalose was removed from the medium by the intact flight muscle. Preliminary results showed that when thorax halves were incubated with trehalose, large amounts of free glucose appeared in the medium. Controls incubated in carbohydrate-free saline showed no glucose accumulation. This effect was probably due to activity of the hydrolytic enzyme, trehalase, recently isolated from *Phormia* by Friedman (1960). We have interpreted our results as an indication that trehalose hydrolysis might be occurring at or near the muscle surface, and a detailed study of this is in progress. The hydrolysis of trehalose complicated attempts to measure the rate of trehalose uptake since several factors were always involved: conversion of trehalose to glucose, uptake of trehalose, and uptake of glucose. These considerations did not undermine the interpretation of the respiratory studies, because a lag in rate would be expected if trehalose first had to be hydrolysed, allowing glucose to reach an effective concentration in the medium, and none was ever observed. Although direct proof is lacking, it is likely that the flight muscles are permeable to trehalose. The only work dealing with trehalose penetration into intact insect tissue is that of Treherne (1960) which showed that this sugar penetrates the cells of the nervous system of *Periplaneta americana*. Permeation of trehalose into muscle is the subject of continuing research.

Blood volume

A knowledge of the blood volume was needed for several aspects of this study, but to our knowledge no such information is available in the literature for *Phormia regina*. Hudson (1958) used 6 μl. as an approximation, derived from information gathered by Beard (1953). We have used dilution of inulin-carboxyl-^{14}C to estimate the blood volume of *Phormia*. This method has been used previously in insects by Levenbook (1958) and by Bricteux-Grégoire & Florkin (1959). A tracer amount of inulin-carboxyl-^{14}C (3625 cts./min. ± 67) contained in 0·524 μl. of water was introduced into the blood by injection. After an appropriate incubation period an aliquot of the blood was removed and delivered directly on to a planchette, which was then dried and assayed for radioactivity. This result permits the calculation of blood volume. The results of these studies, given in Table 4, indicated a blood volume, or inulin space, of about 6–7 μl. for *Phormia* under these conditions. This volume was not altered appreciably by a continuous flight of 30 min., in spite of the probable increase in water influx into the blood from the gut and perhaps other tissues during flight (cf. Hudson, 1958).

Table 4. *Blood volume of* Phormia *estimated from dilution of inulin-carboxyl-*^{14}C *administered by injection*

Time after injection (min.)	No. of flies	Blood volume ± S.E. (μl.)	Range (μl.)
5	5	7·07 ± 0·26	6·64–7·56
10	17	6·72 ± 0·17	6·05–8·81
30	7	6·48 ± 0·42	5·40–8·95
30 (flight)	8	6·67 ± 0·13	5·61–8·45

Analysis of excretory drops collected up to 1 hr. following the injection showed only traces of radioactivity (< 1 % of the amount injected) indicating that the inulin was not being excreted during this time. When whole flies were homogenized in 5 % trichloroacetic acid 1 hr. after the injection of ^{14}C-labelled inulin and the supernatant chromatographed on paper and assayed *in toto* for radioctivity by serial elution, 97 % of the injected dose was recovered from the inulin area of the chromatogram ($R_F = 0$). This observation makes it unlikely that inulin was being metabolized or degraded during this period.

Studies on blood glucose and trehalose

Origin of blood trehalose

As mentioned, *Phormia* utilizes carbohydrate at an estimated rate of about 15 μg./min./fly during continuous flight (Hudson, 1958). Since the blood volume of *Phormia* is about 6–7 μl., the maximal amount of trehalose in the blood under optimal conditions (when trehalose concentration is about 30 g./l.) would be roughly 210 μg. This amount could support continuous flight for about 14 min., while it has been shown that well-fed flies can fly continuously for about 3 hr. (Fig. 1). It is obvious that the amount of trehalose stored in the blood would contribute very little during long flights unless trehalose was continually being secreted into blood. The origin of blood trehalose became, therefore, a question of considerable importance, particularly the rate at which this sugar could be delivered into the blood. The approach to this problem was aided by two sets of observations. Wigglesworth (1949), in a classical study, showed histochemically that substrate for the flight of *Drosophila melanogaster* came in large part from glycogen stored in the abdominal fat body. This glycogen must be mobilized into the blood in some form and transported to the flight muscles. Clements (1955) observed a similar situation using the mosquito *Culex pipiens*. Obviously, blood trehalose could be the transport form of fat body glycogen. A study of fat body was undertaken to determine whether or not this tissue was in fact the site of blood trehalose synthesis. During the course of this study a report by Candy & Kilby (1959) appeared, showing that the fat body of the locust was a site for trehalose biosynthesis, and they have recently described the pathway involved in this synthesis in some detail (1961).

Fat body. Intact fat body was removed from the abdomens of male flies, washed, and incubated in carbohydrate-free saline. Aliquots of the medium were then chromatographed at various times. Development and coloration of such chromatograms showed that large amounts of trehalose appeared in the medium. Experiments were now undertaken to measure the rate of this production. Fat body was prepared and incubated as described, and the medium was assayed quantitatively for trehalose immediately upon placing the tissue in the flask, and again after 1 hr. of incubation. The results of eight experiments showed that trehalose was liberated into the medium at a mean rate of 252 μg. (± 18 S.E.)/mg. dry weight fat body/hr. (range, 184–341). The possibility that this trehalose resulted merely from lysing of the cells with the liberation of preformed trehalose stores was ruled out by the following observations. Freshly isolated fat body was analysed for trehalose content by the methods described for flight muscle. In four separate determinations trehalose was never found to be present at levels exceeding 26 μg./mg. dry weight of fat body, showing that the large amounts of trehalose produced into the medium by the fat body *in vitro* could not be accounted for on the basis of a preformed trehalose store. If these cells were disintegrating, then glycogen, stored in large amounts in this tissue, should also appear in the medium. Since glycogen does not migrate in the solvent systems used, it will be detected by elution of the application zone after the chromatogram is developed. When the base-line of such chromatograms was eluted and carbohydrate determinations carried out, only traces were detected (< 2·8 μg./mg. dry weight fat body/hr.), showing that little if any cell breakage was occurring. It should be emphasized that the fat body of *Phormia* is a very delicate tissue and such incubations must be carried out with caution, particularly when transferring fat body from the wash to the incubation flask. If the 'zero time' reading showed the presence of carbohydrate, or if the medium became opaque during the course of the experiment, that experiment was discarded.

Thus, these results represent a valid measure of the rate at which fat body can synthesize trehalose from endogenous reserves and release it into the medium. Although the same rates need not necessarily occur in the intact animal, it is evident that the fat body would rapidly deplete its store of glycogen during flight if fat body glycogen was the sole precursor. Likewise, blood trehalose should fall to a low level shortly after the onset of flight, instead of decreasing gradually as we have observed. It has been shown that the gut delivers large amounts of dietary glucose into the blood of this fly during flight (Hudson, 1958). In spite of this, the concentration of blood glucose during flight remains fairly constant (Fig. 2), indicating that this sugar is being removed as quickly as it enters the blood. No doubt the flight muscles are utilizing some of this blood glucose. If the fat body could directly utilize blood glucose to synthesize blood trehalose, fat body glycogen might be spared to some extent without removing the supply of trehalose to the flight muscles.

To test this hypothesis we measured the ability of the intact fat body to synthesize trehalose directly from hexoses found in the environment. Mannose, fructose, glucose, and galactose (all uniformly labelled with ^{14}C except galactose which was labelled in the 1-position) were incubated separately with intact fat body (pooled from ten flies) for 30 min., all hexoses being present at a concentration of 200 mg. %. After the incubation period trehalose was isolated from the medium and its specific activity was determined. This result was expressed as a percentage of the specific activity of the hexose added to the medium. This figure, given in Table 5, can be taken as a measure of the ability of fat body to remove these four sugars from the medium, incorporate them into trehalose after the necessary modifications, and liberate this trehalose back into the medium. Clearly, fat body *in vitro* can convert exogenous glucose, mannose, and fructose into trehalose which is then liberated into the medium.

Table 5. *Intact fat body* in vitro; *production of trehalose into the medium from exogenous* ^{14}C-*labelled hexoses*

	Specific activity (counts/min./μg.)		Trehalose specific activity as a percentage of hexose specific activity
^{14}C-hexose in medium	Added hexose	Isolated trehalose	
Glucose	192	63	33
Mannose	210	58	28
Fructose	266	79	30
Galactose	485	22	5

The relatively high specific activity of the trehalose argues against glycogen as a necessary intermediate in this synthesis. Galactose was utilized to a lesser extent, which could be due to the relative inability of the fat body to take up galactose, to convert it into glucose, or both. It is interesting to compare these results to the findings of Wigglesworth (1949) that mannose, fructose and glucose when fed to exhausted flies restored the ability to fly continuously, whereas galactose feeding produced only brief, intermittent flights. Also it might be added that experiments on the fat body of the woodroach, *Leucophaea maderae*, have yielded similar results with respect to trehalose synthesis from labelled sugars added to the medium.

If exogenous glucose was sparing the mobilization of fat body glycogen, then the rate of trehalose production should not be increased by addition of glucose to the medium. To test this we incubated intact fat body with 200 mg. % glucose and measured trehalose production. The results of five experiments showed an average trehalose production rate of 274 μg. (±31 S.E.)/mg. dry weight fat body/hr. (range, 197–310). This figure is not much different from the rate of trehalose production from endogenous reserves. Although a more complete analysis is needed, these results, and those shown in Table 5, indicate that the fat body can synthesize blood trehalose directly from blood glucose, and that this spares, to some extent, the mobilization of fat body glycogen for blood trehalose synthesis.

Next we measured the ability of other tissues to synthesize blood trehalose. The blood, gut, and flight muscles appeared to be possibilities.

Table 6. *Incubation of blood* in vitro *with glucose-U-*^{14}C

For each experiment blood samples were pooled from six flies which had previously been injected with glucose-U-^{14}C. Counting errors at the 1 % level.

Experiment	Incubation period (min.)	Activity (counts/min./μl. blood ± counting error)	
		In glucose	In trehalose
1	0	1179 ± 43	86 ± 13
	5	1140 ± 41	78 ± 11
	15	1161 ± 42	83 ± 12
2	0	1485 ± 47	98 ± 11
	15	1520 ± 54	104 ± 14
	30	1476 ± 53	99 ± 14
3	0	1361 ± 50	30 ± 5
	30	1349 ± 49	35 ± 6
	60	1308 ± 48	39 ± 8

Blood. To test whether trehalose synthesis could occur in the blood itself, 160 μg. of glucose-U-^{14}C having an activity of about 20,000 cts./min. was injected into the blood of three groups of six flies each. This amount will produce approximately a 20-fold increase in the concentration of blood glucose and should upset any equilibrium existing between trehalose and glucose towards trehalose synthesis. Aliquots of the blood were removed about 2 min. after the injection had been made and were pooled from each group for incubation. A known aliquot was delivered on to the base of a paper chromatogram after various incubation periods for determination of radioactivity in glucose and trehalose. The results, given in Table 6, showed no significant change in the distribution of activity between these two sugars, even after 60 min. of incubation, making net trehalose synthesis, or even hydrolysis, a very unlikely possibility. The data also showed that some of the injected glucose was converted to blood trehalose in the brief period *before* the blood was removed, a point to which we will return. The method used in this study permits visual examination of the blood as it is removed from the fly. This is necessary since free-floating fat body cells are occasionally present in the blood. The presence of these cells would obviously alter the results obtained.

Mid-gut. Thes tudies of Treherne (1958*a*, *b*), using the desert locust, showed that dietary ^{14}C-labelled glucose quickly appeared as trehalose in the blood, but no evidence was presented for the site of this conversion. The works of Candy & Kilby (1959, 1961) indicated that the gut of the desert locust, a likely site for this conversion, was not active with respect to trehalose synthesis. Nevertheless, it was possible that the gut of *Phormia* was able to effect the synthesis of blood trehalose, the mid-gut being the most likely region. Accordingly, the mid-gut was removed from ten flies, cut into small pieces, washed, and then incubated in 0·1 ml. of saline containing 500 mg. % glucose-U-^{14}C, having a specific activity of 160 cts./min./μg. Assay of the incubation medium by the usual methods showed essentially no activity in the trehalose area of the chromatogram even after 120 min. of incubation. In another experiment a starved fly was fed on a solution containing 1 % glucose-U-^{14}C (specific activity, 210 cts./min./μg.) marked with carmine. Immediately after feeding, the mid-gut was ligatured just below the proventriculus, and again at the junction of the mid- and hind-gut. This intact piece of mid-gut (coloured bright red) was then removed, washed well, and incubated in 0·1 ml. saline for 1 hr. No leakage at the ligatures occurred since the medium remained clear of carmine. The medium was then chromatographed *in toto*. After development, the entire chromatogram was serially eluted in 2 cm. strips directly on to planchettes which were dried and assayed for radioactivity. The glucose area contained an activity of 34 cts./min. (±6), with essentially all the activity being confined to this area. These results, although rather preliminary, indicate that the mid-gut does not contribute appreciably, if at all, to blood trehalose.

Flight muscle. The trehalase of flight muscle, whose presence was indicated by the *in vitro* studies, could theoretically synthesize trehalose. Although the kinetic studies on trehalase isolated from *Phormia* (Friedman, 1960) indicated that net synthesis was unlikely ($K_m = 6{\cdot}7 \times 10^{-4}$M) experiments were performed to test this possibility. Preparations of intact flight muscle were incubated for 1 hr. in 0·1 ml. saline, and the medium was assayed for trehalose. Small amounts of trehalose were always detected but never exceeded 2·5 μg./mg. dry weight muscle per hour in the eleven cases studied. Likewise, trehalose isolated from the medium in which muscle had been incubated with 500 mg. % glucose-U-^{14}C (specific activity, 210 cts./min./μg.) always showed low levels of radioactivity. Since small amounts of fat body are found in the thorax (Wigglesworth, 1949, and unpublished observations) this tissue rather than muscle might be responsible for the activity observed. In any case, it is apparent that the flight muscles, like the blood and mid-gut, do not play a significant role in the production of blood trehalose in *Phormia*. This view is substantiated by the finding of Turbert & Smith (1961) that the fat body of locusts was the only tissue of those analysed which showed high levels of activity with respect to glucoside synthesis, and by the work of Candy & Kilby (1959, 1961) showing that appreciable trehalose synthesis took place only in the fat body of the desert locust, *Schistocerca gregaria*.

Synthesis of blood trehalose in vivo

There being now good reason to believe that the fat body is the exclusive site of trehalose synthesis, and with the knowledge that this tissue *in vitro* can synthesize and liberate trehalose into the medium directly from exogenous glucose, it was possible to inject ^{14}C-labelled glucose into the blood and to measure blood trehalose synthesis by the fat body *in vivo*.

Accordingly, a tracer amount of glucose-U-^{14}C contained in 0·524 µl. of saline and having an activity of 1349 cts./min. (±35) was injected into the blood of each individual of five groups of flies which had been starved for 24 hr. Each group consisted of at least ten flies. An aliquot of the blood was removed from individuals of one group at 30 sec. after the injection, and these aliquots were pooled on the base of a paper chromatogram. The other four groups were treated in the same manner except that the blood was sampled at 2, 5, 10 and 20 min. after the injection. After development of the chromatograms, trehalose and glucose were eluted, and aliquots of the eluates were taken for chemical and radioactivity determinations. Table 7 summarizes the results of these experiments. The 'zero time' figure was calculated from the known injected dose and the estimated blood volume, and, therefore, is only an approximation. Radioactivity in trehalose was measurable within 30 sec., and increased as the radioactivity in glucose decreased. The percentage of radioactivity appearing in blood trehalose increased very sharply with time, reaching about 50 % in the short period of 2 min., and about 90 % at 10 min. following the injection. Serial elution and assay of entire chromatograms showed that most of the activity was confined to glucose and trehalose (> 96 % of the total recovered activity), but the area between trehalose and the base-line always contained significant levels of activity. These results, coupled with the results of the *in vitro* studies, demonstrated that blood glucose is indeed an immediate precursor of blood trehalose, that this conversion is very rapid, and that fat body is the primary site of blood trehalose synthesis.

Table 7. *Distribution of radioactivity between blood glucose and trehalose at various periods after the injection of glucose-U-*^{14}C *into the blood*

(Counting errors at the 1 % level.)

ubation time min.)	No. flies per group	Activity (counts/min./µl. blood ± counting error)		Percentage of activity in trehalose	Specific activity (× 10³ counts/min./µmole ± counting error)		
		Blood glucose	Blood trehalose		Blood glucose	Blood trehalose	$\frac{\text{Glucose}}{\text{Trehalose}}$
0	(Calculated)	202 ± 5·0	0	0	7482 ± 19	0	—
0·5	11	200 ± 5·2	6·1 ± 0·1	2	386 ± 10	2·3 ± 0·03	167·8
2	10	39·3 ± 2·3	40·7 ± 1·4	50	109 ± 6	13·6 ± 0·5	8·0
5	10	7·0 ± 0·1	42·9 ± 1·5	85	26 ± 0·4	13·3 ± 0·4	2·0
10	10	3·9 ± 0·1	49·1 ± 1·7	90	15 ± 0·3	21·6 ± 0·7	0·7
20	13	1·7 ± 0·1	26·0 ± 0·9	92	12 ± 0·3	10·3 ± 0·3	1·2

In this connexion Winteringham (1958), using the housefly, found that injected glucose-U-^{14}C rapidly appeared as trehalose in extracts of the thorax, and Treherne (1958*b*, 1960) has shown that glucose-U-^{14}C injected into the blood of the roach *Periplaneta americana* and the locust *Schistocerca gregaria* was rapidly converted to blood trehalose. It appears, therefore, that this conversion by the fat body is a common feature of trehalose metabolism in insects, regardless of whether they use fat or carbohydrate as the main source of energy for flight.

Hydrolysis of blood trehalose in vivo

It has been mentioned that considerable amounts of free glucose appeared in the medium when intact flight muscle preparations were incubated with trehalose. The question arises as to whether this actually takes place in the intact animal, or is merely an *in vitro* artifact.

To answer this question we injected each of a number of flies with 3·5 μg. of ^{14}C-labelled trehalose, having an activity of about 4574 cts./min. and contained in a volume of 0·524 μl. At 15 min. after the injection an aliquot of blood was removed from each fly, and the radioactivity in trehalose and glucose determined as usual. The results, given in Table 8, showed that appreciable amounts of radioactivity appeared in glucose, representing up to about 30% of the total radioactivity found in the blood. We concluded that the hydrolysis of blood trehalose did occur. However, the site of this hydrolysis is not at all clear. Locust blood can hydrolyse trehalose (Howden & Kilby, 1956) and Friedman (1960) has reported that the blood of *Phormia* contains a trehalase, the activity of which was demonstrable only after the blood had been diluted *in vitro* (1:100). Our results tend to support the finding that undiluted blood *in vitro* is not active (Table 6), yet we have found that trehalose hydrolysis was occurring in the blood of the intact organism (Table 8). It is unlikely that the small volume injected (0·524 μl.) could dilute the blood enough to activate this trehalase, although such a possibility exists. Noteworthy in this regard is the apparent absence of trehalase activity in the blood of *Leucophaea maderae* (Zebe & McShan, 1959) and the silkworm, *Bombyx mori* (Saito, 1960). Our *in vitro* studies with intact flight muscle indicated that this tissue was responsible for the trehalose hydrolysis observed in the blood under *in vivo* conditions, perhaps by an enzyme found near, or on, the muscle surface. It would appear therefore that the blood and the flight muscles of *Phormia* contain a trehalase. The regulation and function of these two sites of trehalase activity are poorly understood at present.

Diagrammatic representation of carbohydrate transfer between the body compartments

Although based on experimental evidence, the diagram given in Fig. 4 is considerably simplified and even incomplete. Nevertheless, it should serve the function of integrating some of our results and, more important, indicating possibilities for future research. Before discussing this diagram it should be pointed out that a good deal is known concerning the intracellular metabolism of carbohydrate in various insect tissues, and those interested in a survey should consult reviews by Wyatt (1961), Sacktor (1961), Gilbert & Schneiderman (1961), Rockstein (1957), and Gilmour

Table 8. *Distribution of radioactivity between blood glucose and trehalose 15 min. after the injection of ^{14}C-labelled trehalose into the blood*

(Counting errors are at the 1% level.)

Fly number	Activity (counts/min./μl. blood ± counting error)		Percentage of activity in blood glucose
	Blood trehalose	Blood glucose	
1	143·8 ± 10·3	33·7 ± 4·4	19
2	42·4 ± 3·2	18·7 ± 2·4	31
3	382·8 ± 31·4	35·0 ± 4·6	8
4	80·5 ± 6·6	20·8 ± 2·7	19
5	214·1 ± 17·6	69·2 ± 9·0	24
Average	173 ± 14	36 ± 5	20

(1961). However, there are few studies of the transfer of carbohydrate between the body compartments, and the regulatory factors involved in these transfers. This has been one objective of the present study.

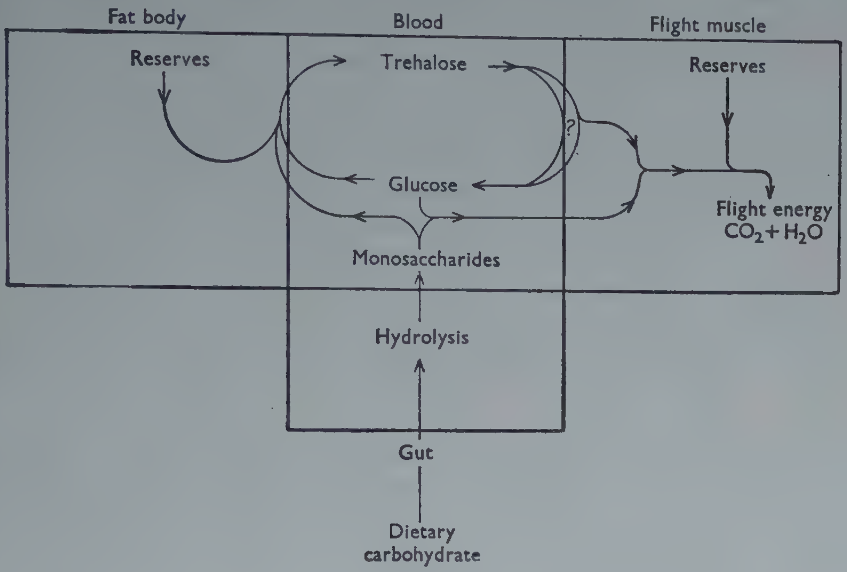

Fig. 4. Diagram of the transfer of carbohydrates between various tissues during flight.

As shown in Fig. 4, dietary carbohydrates after hydrolysis in the gut are absorbed into the blood largely in the form of monosaccharides. These monosaccharides are removed from the blood by various tissues including the fat body. The fat body, and probably only the fat body, can utilize these sugars as well as endogenous reserves for the synthesis of blood trehalose. It will be recalled that this synthesis can occur at a very rapid rate. The factors regulating the partitioning of blood glucose between the synthesis of fat body glycogen and blood trehalose are not known, nor those involved in the mobilization of fat body glycogen for that matter. This channeling of post-absorptive and stored carbohydrates into a single transportable intermediate by the fat body is strikingly similar to the production of blood glucose by the mammalian liver, the difference being of course that insect fat body mobilizes trehalose instead of glucose into the blood. Obviously, the enzymic composition of the fat body will determine which monosaccharides can serve as precursors of blood trehalose. We know that mannose, glucose, fructose, and to some extent even galactose can be converted (Table 5) and there is evidence that the five-carbon sugar fucose cannot be utilized for trehalose synthesis (Evans & Dethier, 1957). It is interesting that fucose has no nutritional value for *Phormia*, while mannose, fructose, glucose and even galactose have considerable nutritional value (Hassett, Dethier & Gans, 1950). These results would predict that the enzymes necessary for the conversion of the above-mentioned hexoses to glucose, and thus to trehalose, are present in the fat body, while those for the conversion of fucose are not.

The subsequent fate of blood trehalose is poorly understood in biochemical terms, but the respiratory study of flight muscle *in vitro*, as well as the wing-beat frequency studies, justify the indication in Fig. 4 that the flight muscle can metabolize this blood sugar to carbon dioxide and water with the concomitant liberation of energy for the flight process. Reserves stored in the flight muscle as well as sugars absorbed from the gut no doubt add to the energy output and we will consider this in more detail later. Blood trehalose can also be hydrolysed to free glucose by enzymes present in the flight muscle and perhaps, under certain conditions, in the blood itself. The question mark in Fig. 4 indicates that the site of trehalase activity might be on or near the muscle surface rather than intracellular. A cyclic process therefore exists in the blood which *must* result in glucose and trehalose being the normal blood sugars of this organism. In this connexion we have maintained flies from eclosion on daily injections of mannose into the blood, and examined the blood chromatographically on the sixth day, 2 hr. after the last injection of mannose had been made. Glucose and trehalose were the only carbohydrates present in detectable amounts.

All these processes are taking place concurrently. The levels of glucose and trehalose in the blood at any given time will therefore be determined by the integration of these simultaneous events. Metabolic control could operate on any of these levels, but the fat body is certainly a very likely spot in view of the regulatory function of blood trehalose. By far the biggest gap in our knowledge concerns the mechanism by which the flight muscle removes trehalose from the blood, and the initial steps in the oxidation of this sugar. It is avoiding the issue to state merely that trehalose is a reserve glucose pool, or that trehalose plays the same role in insects as does blood glucose in vertebrates.

The relative importance of blood sugars and tissue glycogen as energy sources for flight

Most studies concerned with the nature of the flight substrate in the Diptera have concluded that it was glycogen. Glycogen stored in the flight muscle certainly is utilized during flight, and the glycogen content in whole *Drosophila* and the thorax of *Lucilia* have been correlated with the wing-beat frequency (Williams *et al.* 1943). Glycogen stored in the fat body can be mobilized into the blood as trehalose which in turn can supply the active flight muscle, and in this sense fat body glycogen is a flight substrate. The study of Hudson (1958) shows, however, that the amount of carbohydrate depleted exceeds the amount of glycogen depleted during the continuous flight of *Phormia regina*, and the question arises as to the source and nature of this non-glycogen carbohydrate. Table 9 is based chiefly on calculations made from the data of Hudson (1958), and can be used to assess the relative importance of various sources of carbohydrate in *Phormia*. The breakdown of total glycogen in this table has been made on the basis of the relative amounts of glycogen found in the abdomens and thoraces of a large number of well-fed flies after removal of the gut. The average percentage of the total glycogen was found to be about 46% for the thorax and about 54% for the abdomen. Since the flies were all males, most of the abdominal glycogen is in the fat body. Thoracic glycogen is distributed between the flight muscle and thoracic fat body, but mostly in the former. The right-hand column of Table 9 is an estimate of the percentage of the total flight time which a given source could maintain, based on a utilization rate of 15 μg./min./fly. It can be seen that total body glycogen accounts for only about 20% of the total flight time, whereas crop sugar under these conditions would maintain about 69% of the flight duration. Since crop

Table 9. *Evaluation of various tissues as sources of carbohydrate for flight*

(These calculations are based on the data of Hudson (1958). The 'estimated maximal amounts' represent the maximal amounts of carbohydrate (as glucose) found in the various sites following the ingestion of 1·8 mg. of glucose (10 μl. of a 1 M. solution). Flight duration was estimated from a utilization rate of 15 μg./min./fly.)

Site	Estimated maximal amount (μg./fly)	Estimated flight duration on this amount (min.)	Percentage of total flight accounted for
Crop	2790	186	69
Mid-gut	252	17	6
Blood	199	13	5
Total body:			
Glycogen	791	53	20
Abdomen	(427)	(29)	(11)
Thorax	(364)	(24)	(9)

and mid-gut sugar can serve as a flight substrate only after reaching the blood, and since fat body glycogen must be mobilized and transported as trehalose in the blood it can be estimated that about 90 % of the flight duration is maintained by the oxidation of blood sugars, the most abundant of which is trehalose. The quantitative importance of the crop, rarely mentioned in studies of insect flight metabolism, is rather obvious. In this regard there is reason to believe that the crops of blowflies in nature (Evans & Browne, 1960), and even of blood-sucking flies (Hocking, 1953) contain large amounts of concentrated sugar solutions. In fact, Hocking (1953) concluded that crop sugar was the most important source of flight energy for some of these insects. These observations indicate that correlations between glycogen and the flight process must be viewed with caution. For example, a fly which has been flown to exhaustion will fly vigorously for long periods almost immediately after given access to a concentrated sugar solution. In this case, flight energy is obviously derived from the oxidation of blood sugars. Clearly, glycogen is of no consequence here. On the other hand, a fly which is replete with respect to flight muscle glycogen must also have high concentrations of blood trehalose since dietary sugars are common precursors for the synthesis of glycogen and trehalose. As a result it is difficult to evaluate the relative contributions of glycogen and trehalose, under these conditions, during the *initial* stages of flight.

Although the values given in Table 9 are only estimations, they point out that flight muscle glycogen cannot be considered as the major flight substrate in this blowfly during continuous flight to exhaustion, and this might well be the case in many Diptera. Thus, it would appear that blood trehalose, and certain monosaccharides delivered into the blood from the gut, normally provide the main source of energy for the continuous flight to exhaustion of *Phormia regina*.

It should be emphasized that the conclusions derived from this study refer only to the Diptera since quite different processes may be operating in the Orthoptera (Krogh & Weis-Fogh, 1951; Weis-Fogh, 1952), Lepidoptera (Beall, 1948; Zebe, 1954), and Homoptera (Cockbain, 1961) where fat appears to be the chief energy source for flight.

SUMMARY

1. The finding (Evans & Dethier, 1957) that glucose and trehalose are the normal blood sugars of the blowfly *Phormia regina* has been confirmed.

2. It is estimated that most of the flight energy is derived from the oxidation of trehalose and, to a lesser extent, of other sugars found in the blood.

3. Several lines of evidence indicate that the concentration of blood trehalose normally regulates the rate at which energy is expended by the flight muscles during flight. 'Exhaustion' results when trehalose cannot be supplied to the flight muscle at the necessary rate.

4. Fat body is the chief source of blood trehalose; endogenous and exogenous substrates are used for its synthesis. The rate of blood trehalose synthesis can be very rapid, almost compensating for the rate of utilization by the flight muscle during flight.

5. It appears, therefore, that the intensity of flight is determined largely by the interaction of two rate processes: the rate of trehalose utilization (flight muscles) and the rate of trehalose synthesis (fat body).

6. A diagram is presented which accounts for the establishment of glucose and trehalose as the normal blood sugars of this fly and summarizes our findings on the transfer of carbohydrates between various tissues.

7. The blood volume (6–7 μl.) has been estimated from dilution of injected ^{14}C-inulin. This volume is not changed appreciably by flight.

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NUTRITIONAL REQUIREMENTS OF AXENICALLY CULTURED *DROSOPHILA MELANOGASTER* ADULTS

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INTRODUCTION

Although much has been done towards defining the nutritional requirements of immature forms of insects (Dougherty, 1959), very little attention has been paid to the needs of adults. Adults of many species can subsist when fed only simple sugar solutions, and this finding has led to many interesting studies of the suitability of various carbohydrates for the maintenance of adult life (see House, 1961) but also, apparently, to neglect of the examination of the requirements for full adult existence, which in the female involves the production of normal numbers of viable eggs. It cannot be assumed that larval and adult nutritional needs are the same, particularly for a species which inhabits different environments as larva and adult. Further, since ability to produce eggs (and sperm) is of primary significance for survival, it is important that the relationship of nutrition to egg production should be studied more extensively than it has been. The purpose of this paper is to do this for the fruit fly (*Drosophila melanogaster*) whose larval nutritional requirements have already been defined (Sang, 1956, 1959).

Only one of the many previous studies of adult nutrition was carried out using axenic culture techniques (Singh & Brown, 1957), and this showed that the anautogenous *Aedes aegypti* required 'the 10 indispensable amino acids of mammalian nutrition' in order to lay eggs. The vitamins, salts and other nutrients essential for larval growth and development were not needed for egg formation under the conditions of this test. Unfortunately, egg production was measured only during a relatively short period and was at a low level (nine eggs per female in 6 days) so that it is unlikely that stores carried over from the larval stage had been depleted. That such stores do exist in *A. aegypti* is evident from the work of Dimond, Lea, Hahnert & de Long (1956) who found that a dietary supply of histidine and methionine was not essential for the production of the first few eggs laid by females which had been reared under non-aseptic conditions but fed a defined diet as adults. When these stores were consumed an essential requirement for the two amino acids could be exposed. It was also found that cystine was necessary for a high level of egg production. It follows that similar reserves of vitamins and salts, etc., may have been sufficient to allow the production of the small number of eggs reported by Singh & Brown (1957), and that their conclusion that 'vitamins are not required in adult nutrition' is likely to be true only under the conditions of their tests.

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Harlow (1956), who has reviewed much of the literature on adult nutrition, was able to provide evidence for the conversion of the material of the pupal to the adult fat body, and its subsequent transfer to the ovaries at the time of yolk formation in *Protophormia terrae-novae*. By itself this transfer was not sufficient to permit egg formation when sucrose was supplied for the maintenance of the flies, or if protein was also added to the diet. Accessory factors, thought to be B vitamins and salts, had to be fed along with the protein for eggs to be laid. So there is evidence of a range of adult nutritional types extending from autogenous species (of which *D. melanogaster* is one) which will lay some eggs without being fed as adults, to anautogenous species which have apparently simple requirements (like *A. aegypti*) and to anautogenous species which have complex requirements (like *P. terraenovae*). Egg production is not entirely a matter of nutrition since it is also dependent on hormonal control (Clements, 1956); but in so far as fecundity is regulated by nutrition, the assessment cannot be made dependent on the production of *some* eggs. Tests of adult nutritional needs must be carried through under conditions which allow more or less normal levels of egg production, and these tests must be extended in time so as to ensure depletion of stores which may have been accumulated during the previous feeding stage. Further, the eggs laid must be viable, since it is possible that eggs may be laid which are none the less deficient in some normal constituent. An attempt has been made to satisfy these requirements in the experiments herein described.

One further difficulty must also be noted. In Harlow's (1956) work, addition of all the likely B-vitamins and salts to the diet could not substitute for the 'accessory factors' essential for egg formation; some limiting substance or substances not being provided, egg production could not take place. It follows that tests of requirements must start from a complete diet which gives normal production of viable eggs, and proceed by omission of individual constituents. Some earlier experiments have been conducted in the reverse fashion, by finding the effects of additions to an incomplete diet, and in such cases negative results may have no significance if some limiting substance was not provided in the basal diet or along with the additive. Again, since the biochemistry of egg formation is clearly a dynamic process and may involve conversion of one nutrient to another, it cannot be assumed that the minimal diet defined by omission tests is in itself adequate for egg production. It is necessary to test this diet and to find if anything must be added to balance the minimal food. In so far as possible, this has also been done. Whenever a dietary omission apparently affected ovarian development, the ovary was examined cytologically.

MATERIAL AND METHODS

Some of the technical problems of examining adult nutritional requirements have been indicated above. Axenic larvae have to be grown to provide germ-free adults, and two problems which arise in this connexion relate to the influence of nutrition of these larvae upon the accumulation of larval reserves and to the influence of the genotype of the flies upon egg storage, fecundity and viability. A third problem is that of developing a simple technique for feeding adults so that they produce approximately normal numbers of eggs. The exploratory experiments relevant to these points will not be described in detail, but the reasons for the procedure adopted will be indicated, and the routine finally developed will be outlined.

(*a*) *Larval culture medium*

Fourteen nutrients must be supplied to the *Drosophila* larva if it is to develop into an adult (casein, cholesterol, lecithin (which may be replaced by choline), thiamine, riboflavine, nicotinic acid, pyridoxine, pantothenic acid, folic acid, magnesium, sodium, potassium, phosphorus and chlorine. Addition of biotin, ribose nucleic acid (RNA) and fructose accelerates larval development and reduces mortality; while any trace requirements are apparently met by contaminants from the other constituents. The optimum supplies of most of these constituents have been determined (Sang, 1956), but the usual larval diet (Sang's medium C, *ibid.*) contains excess of the vitamins and salts (since this arrangement simplifies preparation of the medium and has no effect on development, whereas excess quantities of the other constituents retard development). As judged by the amount of yolky ooplasm laid down by adults on a protein-free diet, the nutrient reserve of larvae fed on this medium C is about twice that of larvae fed a modified medium in which the vitamins are supplied only in 50% excess of the determined optimum. For this reason, larvae for experiments were reared on this less efficient medium, which is designated medium D in the Appendix.

(*b*) *Egg storage and genotype*

It is well known that copulation stimulates *Drosophila* to oviposit. Males have therefore to be supplied in the adult cultures, and an attempt was made to keep their numbers equal to those of the females throughout the tests. If males died, and could not be replaced, the culture was discarded.

Two highly inbred wild-type strains (Oregon S and Crianlarich-6) and individuals resulting from the reciprocal crosses between them were tested for the effects of protein deficiency on oogenesis (King & Sang, 1958). At the end of 7 days, females from all strains had synthesized 8–11 eggs. However, both the Crianlarich-6 and the hybrids laid most of their eggs at once, whereas Oregon S stored the majority of the eggs in their ovarioles. Again, a test of these three strains on a Mg-free diet produced abnormalities in the ovaries of the hybrids first, indicating that they depleted their stores of this substance more rapidly than flies from the pure lines. Hybrids have a higher fecundity than the pure lines from which they are derived (Gowen & Johnson, 1946), and tests showed that the eggs laid by hybrids were also more viable. Hybrid adults also survived better under the test conditions. For these reasons, most of the work was done using Oregon S/Crianlarich-6 reciprocal hybrids.

(*c*) *Adult culture medium*

The larval food is set in a gel with agar. This method cannot be used for adults since they do not feed efficiently on an agar gel and because the agar is heavily contaminated, particularly with Ca and Mg salts. An attempt was made to replace the agar with 15% purified starch. Egg production was low in females fed under these conditions, and after 2 weeks their ovaries were abnormal in appearance, showing fusion of adjacent chambers, pycnotic areas in the germaria and abnormal development of the follicle cells surrounding the chambers nearest the germarium. Evidently the females were unable to extract certain nutrients from the starch gel at a rate sufficient for normal oogenesis.

Among the many substrates tested cleaned, non-absorbent cotton-wool proved to be the best vehicle in which to supply the experimental media. Cleaning was performed by soaking the cotton in 1% EDTA overnight, washing twice with distilled water, and soaking overnight in alcohol and again in ether. It was found that 0·3 g. cotton would then absorb 5 ml. of medium C (in this case prepared without agar and supplemented with Mg) and that females kept on this medium had a reasonably high productivity (Table 1). Further, an increased concentration of certain nutrients in the adult diet caused a lowering of egg production (Table 1) just as the same increase would have depressed the rate of larval development. The complete larval food medium (medium C) in a cotton base was therefore taken as the standard when assessing the effects of a diet. Ashless floc was used as a base in some salt omission experiments, although it was less satisfactory than cotton since its surface tended to dry out quickly.

Table 1. *Egg production and egg viability of females kept for 4 days on medium C*

Base	No. of females	Average eggs/♀	Percentage inviable eggs
Starch C × 1	6	2·8	88·1
Cotton C × 1	7	32·1	8·0
Cotton C × 1	7	29·8	1·9
Cotton C × 1·5	5	7·6	35·0
Cotton C × 2	5	7·0	25·7

Production is measured as the number of eggs laid during the 24 hr. after removal from the diet, without further feeding. C × 1·5 and C × 2 had the casein, fructose, lecithin, cholesterol and RNA increased to 150 and 200% of that in C × 1.

(*d*) *Technical routine*

Eggs were collected from mass matings of Oregon S × Crianlarich-6 flies, sterilized, and handled as described by Sang (1956). The hybrid larvae which hatched 24 hr. later were transferred in lots of 25 to 2·5 ml. of medium D (see Appendix), previously gelled and autoclaved in ordinary test-tubes which were covered with cylindrical metal caps. The adults which emerged exactly 9 days later were shaken into 6 × 1 in. boiling tubes containing the test diet absorbed in cotton-wool plugs and having a folded strip of ashless filter-paper above the plug to prevent the flies from sticking to the wet cotton. Egg production of individual females was usually measured after 4, 8 and 16 days on the test diet. By 8 days larvae were present in large numbers in the control cultures and, as larvae would interfere with adult feeding (Robertson & Sang, 1944), all flies were transferred to fresh food on the 8th day. It should be noted, however, that this simple procedure had one disadvantage: dietary deficiencies which depressed female productivity tended to leave adult feeding conditions less disturbed through larval tunnelling of the medium than were those experienced by control flies which were characterized by high productivity. Consequently, the 8- and 16-day-old flies from different experiments do not always come from strictly comparable environments. On the other hand, the condition of the developing larvae in the feeding tubes provided a check on their nutritional state and hence of the germ-free condition of the diet provided.

Studies of egg production were initiated by sucking the females from the feeding tubes into 3 × 1 in. vials, which were stoppered subsequently with corks each capped with a disk of charcoal-blackened agar (containing 2 % ethanol and 1 % acetic acid as oviposition stimulants). Each fly was then allowed to oviposit in the dark for 24 hr. at 25° C. (the temperature used for adult and larval culture). The females were then removed, the eggs counted, and the developing embryos given at least a further 24 hr. incubation, after which unhatched eggs were scored. No attempt was made to keep conditions aseptic, and therefore no food was provided to the ovipositing females, since it was found for flies fed *ad lib.* during egg collection that at least 15 % of eggs laid during 24 hr. were formed from food consumed during that same period. Egg counts from typical experiments are given in the tables.

(*e*) *Cytological examination*

Experience quickly showed that the histological appearance of ovaries remained normal so long as females were laying eggs. Consequently females were sacrificed only after they had stopped laying. Feulgen-stained whole mounts were then prepared according to the procedure of King, Burnett & Staley (1957), except that the staining time was standardized at 1 hr. The whole mounts were examined at magnifications ranging from × 6 to × 1400. Abnormalities looked for included modification of the stage distribution of the developing oocytes; pycnosis of oogonia, follicle cells, nurse cells or oocytes; fusion of adjacent chambers; inhibition of yolk synthesis as shown by the absence of oocytes in stages 8–11; subnormal replication of Feulgen-positive material in nurse cell nuclei; atypical numbers of chambers per ovariole; abnormal differentiation of follicular epithelium; and abnormalities in the shape of maturing oocytes or in the production of the chorion. Where reference is made to stage of development of an oocyte, the classification of King, Rubinson & Smith (1956) is used.

EXPERIMENTAL RESULTS

(*a*) *Major food substances*

Of the five main nutrients required by *D. melanogaster* larvae, three are essential for development (casein, cholesterol and lecithin or choline). Surprisingly enough, omission of cholesterol or choline from the adult diet has no significant effect on egg production under the conditions of the test (Table 2). Omission of fructose, which only slows larval development slightly, does reduce egg production, and it should also be noted that by 16 days the great majority of adults died on this otherwise complete diet. Omission of RNA had only a small effect on productivity (see also King & Sang, 1959), whereas it has a more striking effect on larval development than the omission of fructose. That is, adult survival and production of eggs is adversely affected under our conditions by removal of fructose from the diet, whereas cholesterol, choline and RNA may be omitted without notable detriment to the functioning of females, and this would not have been anticipated from their effects on larval development. However, it should be noted here that in these experiments contamination of other nutrients by these substances has not been eliminated (see p. 805), but the amounts present must have been very small since they could not support larval development.

Table 2. *Effects of omission of major nutrients upon fecundity and egg viability*

	After 8 days' feeding			After 16 days' feeding		
Group	No. ♀♀	Eggs/♀	% inviable	No. ♀♀	Eggs/♀	% inviable
Control	8	33·4 ± 3·8	9·8	7	15·6 ± 0·6	42·1
Less casein	5	0	—	—	—	—
Les fructose	10	9·1 ± 3·7	10·0	5	9·6 ± 5·1	10·4
Less choline	9	30·8 ± 5·2	15·3	8	24·4 ± 4·0	39·4
Less RNA	9	37·3 ± 3·1	8·3	9	11·9 ± 3·8	25·2
Less cholesterol	6	20·6 ± 3·4	8·0	3	29·0 ± 4·2	9·0

Standard errors are given for egg production data. Where significant differences of viability are noted in the text they have been judged by the usual χ^2 test; but no great stress has been laid on egg viability, since this criterion may involve the nutritional status of the males as well as the characteristics of the eggs laid (cf. King & Sang, 1959).

As was anticipated, omission of casein rapidly leads to cessation of egg formation, although 8–12 eggs were laid by females during the first few days on the deficient diet. By the end of a week no oocytes in stages 8–11 are found in the ovary. If the females are then fed live yeast, vitellogenesis restarts immediately and about half the ovarioles contain stage 8–11 oocytes within 24 hr. By 3 days females are laying normally (30 eggs per female day), and they continue to do so for at least 2 weeks. The first dozen or so eggs laid are inviable but subsequent eggs are normal (90% hatch). After 16 days on a protein-deficient diet no oocytes are more advanced than stage 3, many chambers are pycnotic, and recovery is much slower. Of eight females examined after subsequent feeding for 3 days on live yeast, four had no oocytes more advanced than stage 6 and, in all, only three stage-14 oocytes were found. The importance of a protein supply for continued egg production by *Drosophila* is evident from these observations, as is also the ability of the flies to recover from any temporary deprivation.

(*b*) *Amino acids*

Replacement of casein by amino acid mixtures led to many difficulties, for the most part apparently due to contamination of these acids by toxic substances. Eventually, reasonable results were obtained using *Sigma* brand amino acids, but even with these there is some evidence that *removal* of particular amino acids (e.g. serine in Table 4) may result in better egg production. There is also a tendency for amino acid to crystallize on the surface of the cotton in a way which would presumably affect adult feeding. However the levels of egg production achieved were sufficient to disclose the effects of omission of single amino acids, all of which were supplied in the natural form.

Table 3 shows in a striking fashion that the ten amino acids essential for the rat are also essential for the adult *Drosophila* female. Omission of any one leads to a reduction of egg output even in 4 days, and to a lowering of the viability of the few eggs laid. Elimination of methionine, arginine, or histidine, even for 8 days, does not result in a complete cessation of egg production, suggesting that the former can be met in part by synthesis of methionine from cystine, and that arginine and histidine may also be slowly synthesized, as has been found with other organisms (Meister, 1957). After 8 days of feeding on diets lacking single 'non-essential' amino acids, no reduction of egg output is evident, nor is infertility notably increased (Table 4).

Table 3. *Effects of omission of 'essential' amino acids upon fecundity and egg viability*

Group	After 4 days' feeding			After 8 days' feeding		
	No. ♀♀	Eggs/♀	% inviable	No. ♀♀	Eggs/♀	% inviable
All amino acids	5	34·0 ± 6·5	0·5	19	48·1 ± 2·0	40·4
Less arginine	7	5·7 ± 0·6	47·5	16	3·1 ± 0·5	76·0
Less histidine	8	4·9 ± 1·4	18·0	7	5·3 ± 2·3	62·0
Less isoleucine	10	0·9 ± 0·2	66·0	10	0	—
Less leucine	6	0·8 ± 0·3	20·0	7	0	—
Less lysine	10	1·0 ± 0·4	20·0	14	0	—
Less methionine	16	15·0 ± 1·2	3·8	11	4·8 ± 1·6	35·8
Less phenylalanine	8	1·8 ± 0·7	7·1	13	0·5 ± 0·3	71·5
Less threonine	8	1·9 ± 0·7	25·0	15	0	—
Less tryptophan	8	6·9 ± 0·9	21·8	7	0·3 ± 0·3	100·0
Less valine	7	1·7 ± 0·7	25·0	10	0	—
Casein medium	9	53·8 ± 3·7	0·5	—	—	—

The composition of the amino acid mixture used is given in the Appendix: the other constituents are the same as those of medium C.

Some significant differences appear by 16 days: notably, the lowered fecundity on cystine-deficient diets and on diets without aspartic or glutamic acids. The first observation might be explained by assuming that the amount of methionine provided in the essential amino acids is insufficient to meet requirements by conversion to cystine (Meister, 1957), and the heightened infertility of the eggs laid would support this assumption. Effects of omission of aspartic or glutamic acid might be a consequence of insufficiency of these non-essential amino acids which are involved in the formation of such key metabolic intermediates as α-ketoglutarate. Direct proof of this possibility would depend on quantitative substitution tests which have not been attempted. It is also interesting to note that elimination of tyrosine affects only hatchability, implying that this substance is synthesized at an inadequate rate from the phenylalanine supplied.

Table 4. *Effects of omission of 'non-essential' amino acids upon fecundity and egg viability*

Group	After 8 days' feeding			After 16 days' feeding		
	No. ♀♀	Eggs/♀	% inviable	No. ♀♀	Eggs/♀	% inviable
All amino acids	10	37·0 ± 3·2	0·8	8	30·0 ± 4·2	17·3
Less alanine	9	34·6 ± 2·7	2·3	8	41·5 ± 3·1	16·6
Less aspartic acid	10	44·4 ± 4·1	2·4	7	18·3 ± 4·3	10·1
Less cystine	8	36·6 ± 2·6	5·1	5	16·6 ± 3·6	37·4
Less glutamic acid	10	54·0 ± 4·7	2·2	7	17·3 ± 7·3	21·5
Less glycine	10	52·7 ± 2·4	4·0	8	24·3 ± 6·0	19·1
Less proline	6	29·2 ± 3·6	8·0	5	27·0 ± 4·6	19·3
Less serine	10	48·5 ± 2·2	6·6	9	42·2 ± 2·0	7·9
Less tyrosine	10	37·3 ± 2·4	3·5	9	39·1 ± 4·2	31·4
Live yeast	10	49·5 ± 2·0	3·4	10	55·3 ± 2·3	1·4

These consequences of the removal of particular non-essential amino acids from the diet imply that essential amino acids alone should not be sufficient to give normal egg production, contrary to Singh & Brown's (1957) finding with *Aedes*. That this is

Table 5. *Effects of additions to the mixture of essential amino acids upon fecundity and egg viability*

Group	No. ♀♀	Eggs/♀	% inviable
Essential amino acids	10	4·8 ± 1·7	25·0
Plus glycine	11	17·2 ± 3·6	54·5
Plus glycine and glutamic acid	9	0	—
Plus glycine and glutamic acid and cystine	14	15·6 ± 3·1	48·6
Live yeast	7	24·4 ± 1·6	2·9

Amino acids were provided in the amounts used in the preceding experiments. Egg production was measured after 16 days.

so is shown in Table 5. Addition of glycine improves output, demonstrating that a non-essential amino acid is necessary for good egg production. The further addition of glutamic acid results in no eggs being laid, but the ovaries of flies from this third group appear to be normal, and contained stage 14 oocytes. The addition of cystine then releases this inhibition of egg laying, indicating the complexity of the balance of non-essential amino acids required for full egg production. This problem was not explored further since it clearly involved quantitative as well as qualitative aspects of the dietary supply of both essential and non-essential amino acids, and these could not be adequately handled with the techniques described.

(*c*) *Vitamins*

All the B vitamins, except possibly B_{12}, are necessary for optimal larval growth (Sang, 1956), and the same qualitative requirement would be expected for egg production. In fact, sufficient larval reserves are carried into adult life to permit the laying of a considerable number of viable eggs, so that the adult requirement is clearly dependent on the status of the larval supply of each vitamin. Under the experimental conditions defined above, the effects of depriving adults of one or other of the vitamins can readily be shown (Table 6), and the results, except for biotin, are as anticipated. Since none of the media contained vitamin B_{12}, it is clear that this vitamin is also not required, except in the trace amounts which may be present as contaminants of the other dietary constituents. Biotin requirements for larval development are conditional on the protein supply, suboptimal protein reducing the requirement (Sang, 1959). So it is possible that the different metabolism of protein involved in egg formation permits normal oogenesis in the absence of this vitamin. It is perhaps worth noting that no larvae grew beyond the second instar from eggs laid in the biotin-deficient medium.

Table 2 shows that omission of RNA has no effect on egg production (see also King & Sang, 1959), whereas removal of the vitamin (folic acid) most involved in the synthesis of RNA lowers both fecundity and egg viability. Diets without both RNA and folic acid permit the same fecundity as those without folic, but the viability of the eggs laid is then lowered (King & Sang, 1959), as might be expected from the known (Travaglini, Levenbook & Schultz, 1958) high nucleic acid content of *Drosophila* eggs.

These results agree with expectation, but as they relate essentially to one end product (nucleic acids) they do not indicate possible interactions involving more than one

Table 6. *Effects of omission of single vitamins from the adult diet upon fecundity and egg viability*

	After 8 days			After 16 days		
Group	No. of ♀♀	Eggs/♀	% inviable	No. of ♀♀	Eggs/♀	% inviable
Control	10	40·2 ± 2·8	1·2	7	32·3 ± 3·4	5·7
Less thiamine	10	13·1 ± 2·5	89·3	4	0·3 ± 0·2	100·0
Less riboflavine	9	6·0 ± 1·7	81·6	7	0	—
Less nicotinic acid	10	12·0 ± 2·1	24·2	—	—	—
Less pyridoxine	6	14·8 ± 3·2	89·0	9	6·6 ± 2·3	100·0
Less pantothenic acid	10	22·6 ± 1·0	17·7	6	3·2 ± 1·3	84·0
Less folic acid	9	5·4 ± 2·2	77·5	—	—	—
Less biotin	9	37·6 ± 3·3	13·3	6	29·7 ± 5·3	21·3
Live yeast	6	52·2 ± 4·1	22·6	10	47·8 ± 2·7	3·4

Table 7. *Effects of removal of pyridoxine, folic acid and RNA on fecundity and egg viability*

	After 4 days			After 8 days		
Group	No. ♀♀	Eggs/♀	% inviable	No. ♀♀	Eggs/♀	% inviable
Control	4	29·5 ± 3·4	0	5	52·6 ± 2·5	15·0
Less pyridoxine	8	13·4 ± 2·3	72·9	10	5·5 ± 2·3	72·7
Less folic acid	6	23·8 ± 2·1	25·2	10	17·8 ± 5·8	62·9
Less pyridoxine and folic acid	5	10·0 ± 1·8	70·0	7	9·9 ± 1·9	76·8
Less pyridoxine and RNA	8	20·3 ± 1·9	63·0	10	27·9 ± 2·7	83·5
Less all three	8	8·4 ± 2·5	90·0	3	12·7 ± 5·0	81·6

reaction system. The effect of omission of pyridoxine concurrently with folic acid, or with RNA, or with both, was examined as an example of the consequences of interfering with more than one system. As before (Table 6) omission of pyridoxine has a more drastic effect than omission of folic acid (Table 7). When both are left out, the consequences are indistinguishable from the effects of not supplying pyridoxine. Omission of pyridoxine and RNA, on the other hand, results in an increase of egg output over that found for omission of pyridoxine alone. Omission of all three substances has the same effect, except that egg viability is lower, as omission of pyridoxine or pyridoxine and folic acid. There is no obvious explanation of the anomalous pyridoxine–RNA result, but the point was not explored further, since this would have involved quantitative as well as qualitative manipulations too laborious to be attempted at this time. The experiments are quoted to show that multiple deficiencies will not necessarily have predictable results and to indicate how natural shortages, which will rarely be limited to a single dietary constituent, may have unpredictable consequences.

(*d*) *Anions and cations*

As with the vitamins, different relative amounts of salts seem to be carried over to the adult stage, and depletion times on deficient diets are consequently different. Even after 8 days there is little evidence that either Na or Ca is necessary for the production of normal eggs, whereas the K and Mg deficiencies result in reduction of egg output before this time, and in mortality of the adults. After 16 days the effect

Table 8. *Effects of omission of some anions and cations upon fecundity and egg viability*

	After 8 days			After 16 days		
Group	No. ♀♀	Eggs/♀	% inviable	No. ♀♀	Eggs/♀	% inviable
Control	9	41·7±3·6	12·0	10	18·3±3·0	31·3
Less K	13	1·6±0·4	100·0	—	—	—
Less P	10	28·1±3·8	29·5	9	20·6±3·4	61·2
Less Ca	7	33·3±4·2	20·5	5	14·6±4·0	54·8
Less Mg	15	7·0±1·4	24·7	13	8·3±2·9	28·5
Less Cl	13	30·4±2·9	40·8	18	22·2±1·6	54·8
Control	8	20·1±2·4	1·0	1	28	0
Less Na	9	27·9±4·1	7·2	2	0	—
Yeast	5	47·6±2·6	4·2	6	38·3±7·5	3·5

Ashless floc was used in place of cotton as a base, Ca-free casein in place of vitamin-free casein, choline in place of lecithin, the anions and cations removed from the vitamins with resins and the RNA replaced with appropriate amounts of adenosine and cytidine. 'Tris' buffer was substituted for the usual phosphates.

of the deficiencies of Cl and P on viability becomes more obvious, even though P was present in a substantial amount as a contaminant of the casein. In the case of sodium the deficiency eventually causes the death of most adults. It is also interesting to note that the Na and Ca deficiencies do not completely inhibit the development of larvae hatching from eggs laid on the food medium, since in both cases a few pupae were found in the second series (i.e. 8–16 days) of cultures (Table 8).

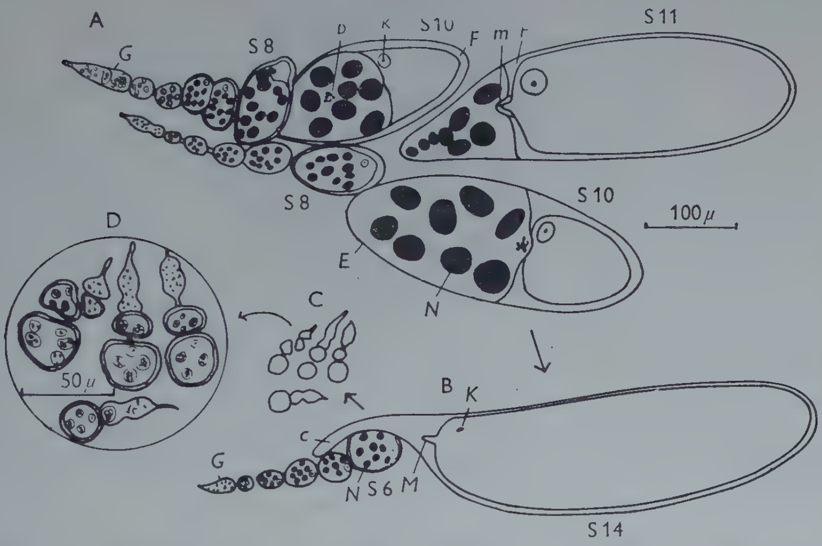
(*e*) *Cytological changes*

The majority of the dietary deficiencies described above produced no degenerative cytological changes in the ovaries, demonstrable with the techniques employed, even when females had ceased to lay. Such ovaries were deficient in oocytes which were in vitellogenic stages, but the structure of those egg chambers present was normal (see Text-fig. 1 B). This result is not surprising in view of the notable homeostatic ability of this organ, as demonstrated by its recovery after prolonged protein deprivation (p. 798) or after aminopterin poisoning (King & Sang, 1959). Put another way, the general response to a particular nutritional deficiency is cessation of oogenesis, not the production of abnormal, and therefore useless, eggs; and this may be an adaptation to the ephemeral character of the natural breeding and feeding sites of the fly. Three exceptions were found to this generalization, and they deserve recording (Sang & King, 1959).

(1) *Deficiencies of essential amino acids.* Omission for 8 days of any essential amino acids other than arginine, histidine or methionine results first in the disappearance of yolk-containing stages (Text-fig. 1 B, cf. 1 A) and then to the disappearance of all stages later than stage 4 (Text-fig. 1 C). As there is no evidence that any chamber can revert to an earlier stage, the assumption must be that stages 5 and higher must develop to stage 14 oocytes and that these are laid as inviable, but superficially normal, eggs. When the ovary has degenerated to this degree and only two to three chambers can be found in each ovariole, it is clear that the structure of the germaria is then also affected (Text-fig. 1 D). No germarial cysts are present, few if any mitoses can be identified, and there is little or no cellular differentiation. The ovaries of flies fed only

on sugar-water show an identical structure after 18 days; thus protein is required for continued proliferation of chambers from the germarium, as well as for vitellogenesis.

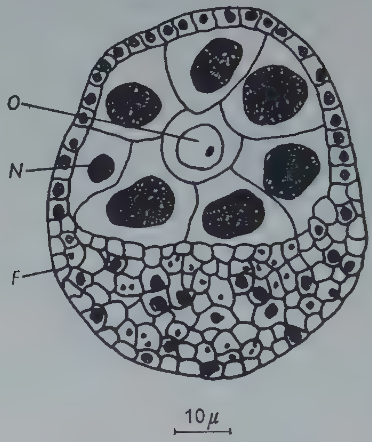
(2) *Pyridoxine deficiency.* Pyridoxine is the only vitamin whose deficiency produces abnormal development of cysts, although it is clear from earlier work (King, 1958; King & Sang, 1959) that analogues of other vitamins may be fed at levels which will lead to pathological conditions. As with other vitamin deficiencies, lack of pyridoxine leads first to a disappearance of yolky stages. Later there is an abnormal development of the nurse cell nuclei (Pl. 1, fig. 1) which is characterized by an increase of the size of the nucleus relative to the stage of development, typically one stage larger, and by an apparent decrease of the Feulgen-positive material in the nucleus. Pl. 1, fig. 1 (right) shows that the chromosomal material is more diffuse in pyridoxine-starved nurse cells, and there is also a notable increase in the number of pycnotic nuclei in the follicular epithelium. Occasional cysts also show displacement of the oocyte to a central location and retardation of the development of some nurse-cell nuclei (Text-fig. 2). In the example shown, there is also an hypertrophy of the follicular epithelium into a localized mass containing many pycnotic cells.



Text-fig. 1. The changing cytological picture occasioned by omitting any essential amino acid from the diet. A. Two normal ovarioles (drawn from Feulgen-stained whole mounts) showing oocytes in stages of active vitellogenesis (S 8, 10 and 11). B. An ovariole at the first phase of deficient development, in which only pre-yolk stages and mature eggs are present. C and D. A later phase in which only two to three chambers are present, none more advanced than S4. Germaria then contain no germarial cysts, show no mitoses and have little cellular differentiation. Flies fed on sugar water have ovaries as in C and D at or before 18 days. *E*, squamous epithelium; *F*, follicular epithelium; *G*, germarium; *K*, karyosphere of mature oocyte; *k*, karyosome of oocyte nucleus; *N*, nurse cell nucleus; *b*, border cells; *c*, chorionic horn; *M*, micropylar apparatus of mature egg; *m*, micropylar cap formed by border cells; S, stage designation. No correction made for shrinkage occasioned by histological manipulation.

(3) *Magnesium deficiency.* Depriving females of dietary magnesium lowers the frequency of oocytes in active vitellogenesis, but it does so more slowly than removal of an essential amino acid. Replication of Feulgen-positive material is retarded in the

nurse cells and no cysts of normal appearance more advanced than stage 6 are found (Pl. 1, fig. 2). However, chambers continue to be produced by the germarium so that an ovariole may eventually contain 11–13 oocytes, of which the posterior 6–8 will be made up of degenerating stage 6 or 7 oocytes and the anterior 5 will be normal stages 1–5. Presumably the degeneration of the nurse nuclei leads to the failure of vitellogenesis, and the breakdown of the Feulgen-positive material possibly permits some re-cycling of the magnesium to the germarium and to the oocytes in early stages of development.



Text-fig. 2. Camera lucida drawing of a stage 8 chamber from a pyridoxine starved female. The oocyte (*O*) is centrally located and a nurse cell (*N*) has an abnormally small nucleus. The follicular epithelium (*F*) has hypertrophied in a localized area and contains many pycnotic cells (cf. Pl. 1, fig. 1).

DISCUSSION AND CONCLUSIONS

These results show that the qualitative requirements of the adult female *Drosophila* are similar to those of the larva, as would be expected since the viable egg must contain everything necessary for the formation of the larva which hatches from it. A difference would be expected only if the adult had synthetic abilities absent in the larva or if the larva had particular requirements for its growth and differentiation. Tests carried out with adults kept for only a few days on restricted diets would often be insufficient to expose these full nutritional needs, for the data presented clearly show that reserves, accumulated during larval feeding, can be drawn from adult tissues and incorporated in the forming eggs. Although the procedures used (p. 796) set a limit to the accumulation of reserves, it cannot be assumed that equivalent amounts of each dietary component would be stored, since the limiting quantity of any one necessary for normal larval development may represent only the maximum required at a particular stage of larval growth. What is carried into the pupa may have no direct relationship to the amounts provided to the larvae, so nothing can be deduced from the data concerning the quantitative nutritional requirements of the adult, except in the broadest terms.

Availability of a protein supply is the requirement most critical for egg production, and ability to recover from protein deprivation is one of the most interesting facts disclosed in these studies. The data (Tables 3 and 4) suggest that this is due to the rapid depletion of reserves of the essential amino acids, other than methionine, arginine and histidine which may be slowly synthesized but at a rate which is insufficient to allow normal egg production. Neither whole protein nor any essential amino acid seems necessary for continued adult existence as such, at least within the limits of the tests described.

Table 9. *Effect of addition of 0·03% cholesterol to a cholesterol-free medium*

	With cholesterol ($n = 9$)	Without cholesterol ($n = 14$)
No. of eggs/female	10·0 ± 3·0	1·79 ± 1·1
Proportion inviable	12·2 ± 3·4	16·00 ± 2·4

Egg production was measured after 14 days of feeding.

The most surprising difference between adult and larval nutritional needs is shown by the apparent ability of females to produce large numbers of viable eggs when cholesterol is omitted from their diet. Cholesterol is essential for larval development (see Clark & Bloch (1959) for comments concerning other species), and no larvae grew beyond the second instar in the adult culture tubes, indicating that the medium was truly deficient in this substance (at least in terms of concentrations necessary for larval development). Chemical tests on large samples of the medium showed that only a small amount of cholesterol or cholesterol-like substances could be present (less than 0·2 μg. per 5 ml. medium). But the amount in eggs laid by yeast-fed females is also low (35 μg. of cholesterol and cholesterol-like substances per g. dry weight) so it seemed possible, if improbable, that contamination might provide sufficient sterol to allow continued egg production. An attempt was made to check this point by careful extraction of medium constituents (Table 9) with warm alcohol and ether prior to compounding them. The medium was unsatisfactory as a result of this treatment, as shown by low fecundity even when cholesterol was added. It was apparently sterol-free, and this resulted in a significant reduction of egg output when compared with the same medium containing added cholesterol.

This example illustrates the difficulty of ensuring that trace amounts of a constituent omitted from the diet are not being provided by contaminants present in the remainder. Consequently, it cannot be concluded that biotin is not required by the adult female (Table 6), but only that if there is a requirement it must be very low. And the same must be said for vitamin B_{12} (which was not tested, since it was known to be present in trace amounts) and for choline.

For the reasons already noted it is impossible to compare the quantitative requirements of adults and larvae with any accuracy. One calculation is worth making: a larva consumes 3 to 5 times its own weight of yeast during its 5 days of growth (Chiang & Hodson, 1950) and an adult eats about its own weight per day (King & Wilson, 1955), so that in both cases we have about the same average daily intake. Yet the level of cholesterol required by the larva is about 1500 μg./5 ml. of medium; whereas the adult needs not more than 0·2 μg. to produce her daily quota of eggs. Obviously the magnitude of the requirements for larval development and for production of viable eggs are of quite different orders in so far as cholesterol is concerned, and probably the same is true for choline and biotin. This kind of difference might be expected since an egg takes 72 hr. to form and does so in the presence of the enzyme systems of the female; i.e. it is supplied with constituents preformed by the mother (King, 1960). It follows that the quantitative nutritional requirements for egg production should be different from those for larval growth.

This difference is further illustrated by the fact that removal of RNA has no effect on egg production; whereas it slows larval growth, since the larva's ability to synthesize adenylic acid is rate-limiting (Sang, 1957). This cannot be the case for egg production, but it apparently can be made so by omission of folic acid. RNA cannot substitute for a folic deficiency as it can in the larva by providing the product in place of the enzyme systems which form it. So either RNA is not used or the rate-limiting process for egg production, involving folic acid, is different from the rate-limiting process for larval growth. Since a great amount of DNA is formed by the nurse cells during the development of an ovum (Jacob & Sirlin, 1959), it seems possible that the rate-limiting process is in the folic-mediated synthesis of DNA. This would imply that DNA is not formed from dietary RNA, which agrees with the fact that it has been impossible to show that dietary DNA spares RNA (Sang, unpublished; cf. House, 1961). The situation is further complicated by the observation that sulphanilamide depresses DNA replication within the nurse cell nuclei (Sang & King, unpublished) implying that the adult can synthesize folic acid to a limited extent.

Failure of nurse cell nuclei to become polyploid seems to result in abnormal vitellogenesis and the formation of abnormal eggs (King & Sang, 1959; King & Burnett, 1957; King, 1959). In this situation it is easy to see that a deficiency of Mg, which is necessary for the integrity of the chromosome, would result in the failure of egg development. The abnormalities of chromosome structure induced by pyridoxine deficiency would be expected to have similar results. What is perhaps more surprising is that the ovary can adapt itself to other deficiencies by suspending its activity, but only after a phase during which abnormal (but not cytologically identifiable by the techniques employed) and inviable eggs are laid. That is to say, although a few inviable, and sometimes deformed, eggs are laid when the diet is deficient in some constituent, the ovary preserves its function by a restriction of the formation of pre-yolk stages. This would be the automatic consequence of a system which partitioned nutrients in favour of the yolk-forming stages, and it seems as if the most posterior oocyte in a chamber is given priority in this respect.

SUMMARY

1. A technique is described for the culture of germ-free *Drosophila* adults on defined diets. The complete larval diet, with the agar base replaced by cotton-wool, was found to be adequate for adults and permitted them to lay more or less normal numbers of eggs during a 16-day test period.

2. Omission tests showed that casein, the B vitamins (other than B_{12} and biotin) K and Mg were essential for normal ovary development.

3. Casein could be replaced with an amino acid mixture. The ten 'essential' amino acids were all found necessary for egg production but arginine, histidine and methio-

nine were apparently synthesized, although at an inadequate rate. The remaining essential amino acids seemed to be the nutrients required in the greatest amounts since egg production stopped soonest when they were omitted. Individual 'non-essential' amino acids could be removed from the mixture, but a supply of them was necessary for normal egg production and viability.

4. Omission of fructose lowers egg production but does not cause its cessation even after 16 days.

5. The difficulty of determining that essential supplies are not being met by contaminants is illustrated by examination of cholesterol requirements. In this case, and possibly also for choline, the requirement for egg formation is very much less than for larval development, and might be satisfied from contamination of the medium constituents.

6. Neglecting these trace supplies (which can be measured only in fractions of a microgram per 5 ml. medium) RNA, choline, cholesterol and biotin seem unnecessary for egg formation. The quantitative requirements of the normal adult female must therefore be different from those of the larva.

7. It was not possible to produce a true P deficiency, or to be certain that traces of Ca and Cl in the medium were not sufficient to permit normal fecundity.

8. The ovary is shown to be capable of recovery from protein starvation and to respond to omission of single essential amino acids by ceasing to form new chambers. The majority of deficiencies result in inhibition of vitellogenesis, after a period when inviable eggs are laid. Only Mg and pyridoxine omission produced distinguishable pathological changes, which are illustrated.

Thanks are due to Dr A. W. Greenwood, C.B.E., for generous hospitality to one of us (R.C.K.) and to Dr W. McIndoe for the sterol analyses quoted above.

APPENDIX

Composition of media employed

Nutrient	Medium C (p.p.m.)	Medium D (p.p.m.)	Amino acid mixture	(p.p.m.)
Casein (low vitamin)	55,000	55,000	Arginine HCl	3025
Fructose	7,500	7,500	Histidine	1650
Lecithin	4,000	4,000	Isoleucine	3850
RNA	4,000	4,000	Leucine	4675
Thiamine HCl	2	0·3	Lysine HCl	4400
Riboflavine	10	1·2	Methionine	1650
Nicotinic acid	12	1·6	Phenylalanine	3025
Ca pantothenate	16	2·4	Threonine	3025
Pyridoxine HCl	2·5	0·1	Tryptophane	1100
Biotin	0·16	0·025	Valine	3850
Folic acid	3	0·3	Alanine	3300
Na_3HCO	1,400	1,400	Aspartic acid	3300
KH_2PO_4	1,830	1,830	Cystine	1100
Na_2HPO_4	1,890	1,890	Glutamic acid	8525
$MgSO_4$	810	—	Glycine	1650
NaCl	130	—	Proline	4400
			Serine	4400
			Tyrosine	3300

Medium C has the same composition as the standard larval culture medium (Sang, 1956) except that agar is omitted and $MgSO_4$ and NaCl added to allow for the quantities present in agar. When lecithin is replaced by choline it is used at 50 p.p.m. Medium D was set with 3% Kobe no. 1 agar. Amino acids were neutralized as necessary and kept as individual stock solutions: the concentrations listed are those used in replacement of the casein. For P deficiencies lecithin was replaced by choline, RNA was replaced by adenosine (925 p.p.m.) and cytidine (460 p.p.m.) and an organic buffer replaced the phosphates. For the Ca deficiencies a calcium-free casein and pantothenic acid were used. In the case of sodium and potassium deficiencies organic buffers were used. For Cl deficiencies Cl-free pyridoxin and thiamin were prepared.

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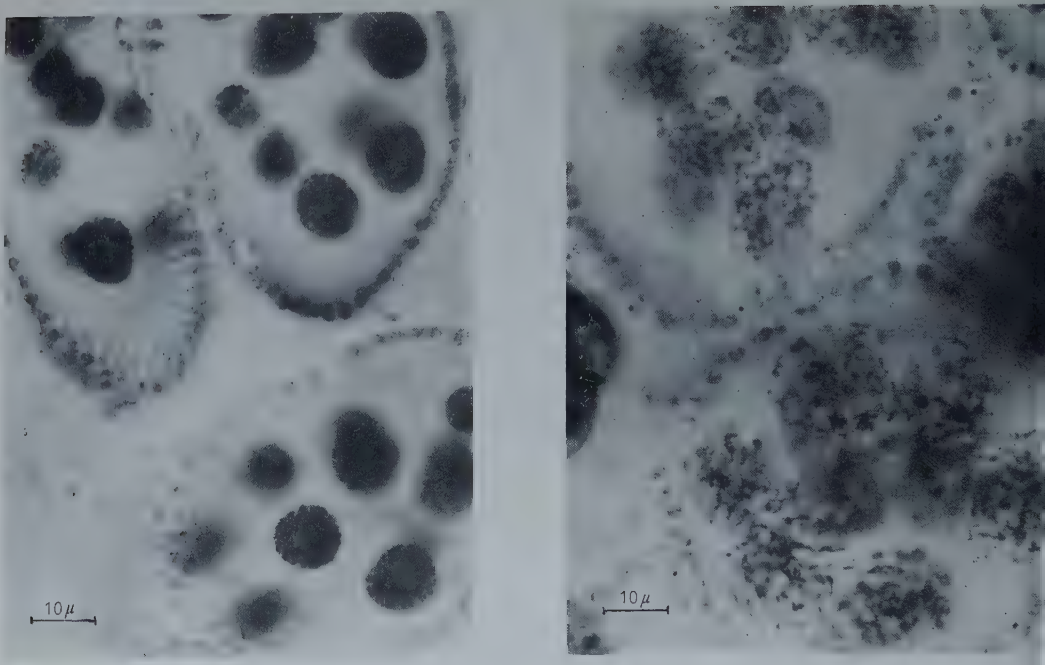

Fig. 1

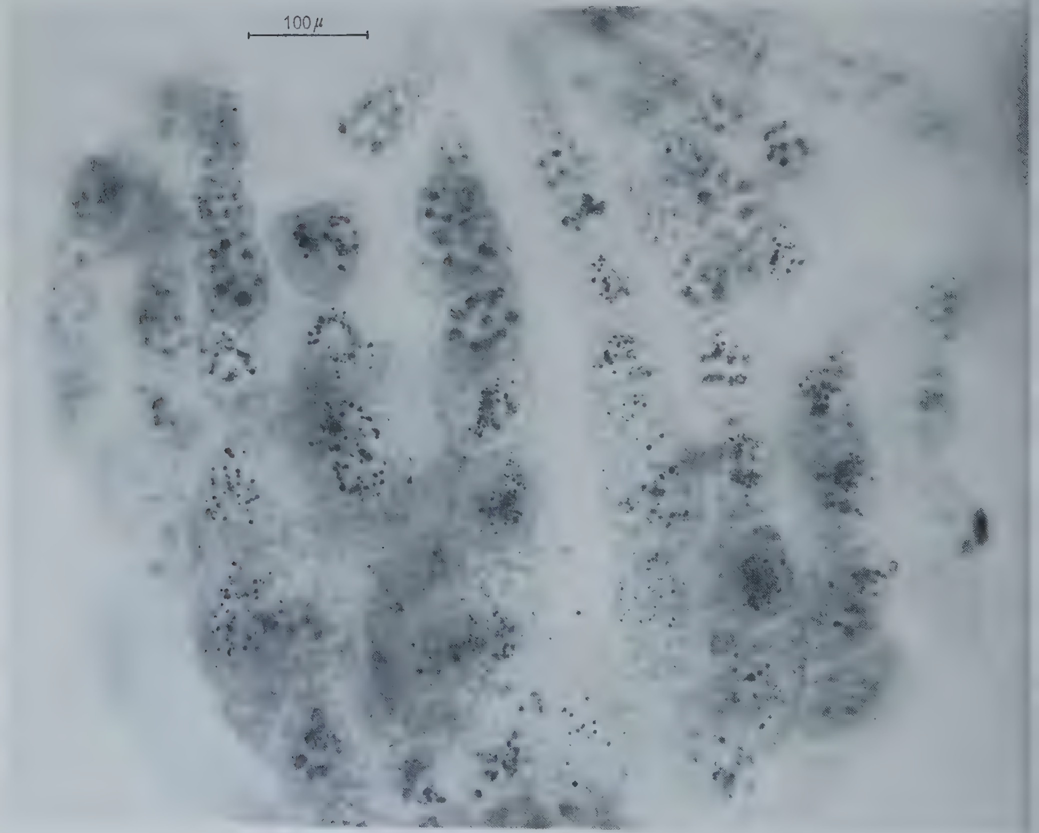

Fig. 2

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(*Facing p.* 809)

Travaglini, E. C., Levenbook, L. & Schultz, J. (1958). Nucleic acids and their components as affected by the Y chromosome of *Drosophila melanogaster*. II. Nucleotides and related compounds in the acid soluble fraction of the unfertilized egg. *Exp. Cell Res.* **15**, 62–79.

EXPLANATION OF PLATE

Fig. 1. Photomicrographs of cysts from a normal (left) and pyridoxine-deficient ovaries (right). The abnormal chambers are equivalent in volume to stage 8, but the diameters of the nurse cell nuclei are characteristic of the next stage. The nuclei contain far fewer Feulgen-positive strands than normal, and they are less compactly organized. Note also that the epithelium around the follicle is characterized by pycnotic areas never found in the controls.

Fig. 2. Preparation of an ovary from a female deprived of Mg for 16 days. The chambers have continued to proliferate and as many as a dozen may be found in an ovariole (cf. Text-fig. 1). None develop beyond stage 6 (pre-vitellogenesis), and the nurse cell nuclei are deficient in Feulgen-positive material. The posterior half of an ovariole is composed of degenerating cysts.

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CENTRES FOR TACTILE AND VISUAL LEARNING IN THE BRAIN OF OCTOPUS

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INTRODUCTION

Octopuses can be taught to discriminate between things that they see, and many experiments have been made to test the effect of brain lesions on performance in visual discriminations. It appears that the optic, superior frontal and vertical lobes together constitute a mechanism for learning to recognize situations by sight (references, see Young, 1961). The whole of this system can be removed without abolishing the capacity to learn to recognize objects by touch; and still further extension of the lesion, beyond the limits of the visual learning system, has no effect upon tactile discrimination until the inferior frontal or subfrontal lobes are included, whereupon touch learning fails (Wells, 1959*b*). From this it would appear that visual and tactile learning are carried on in separate parts of the brain. The experiments reported so far do not, however, eliminate the possibility that with the visual system intact, an octopus might still be able to learn tactile discriminations after removal of the inferior frontal system. The present account shows that tactile learning can be abolished by lesions limited to the inferior frontal system and that such lesions do not prevent visual learning.

MATERIAL

Octopuses of between 250 and 1000 g. from the Bay of Naples were caught and kept as described by Boycott (1954). Parts of the supra-oesophageal brain mass were removed before, or during, the course of training experiments. Most of the animals used in tactile experiments were also blinded by peripheral section of the optic nerves. Operational techniques are described in Wells & Wells (1956, 1957*b*).

In the text, figures and tables, individual octopuses are identified by a number, consisting of a prefix, A, B, C, ..., F indicating the year in which the experiment was made (1954–59, respectively) and a yearly serial number.

Brain lesions were checked from serial sections. Lobes of the brain mentioned in the text are shown in Fig. 1, part of which is also used as the standard diagram in which lesions are plotted for Figs. 2 and 3. Some further details of the anatomy of the inferior frontal system are given in Wells (1959*b*).

IMMEDIATE EFFECT OF BRAIN LESIONS

The lesions made for the experiments to be described do not prevent octopuses from moving about in a normal manner, and it is generally only when the animals are trained that it becomes apparent that they are in any way different from controls.

An exception is that animals blinded by section of the optic nerves or removal of the optic lobes tend to move about their aquaria with the arms more outspread than usual, cease to use the 'home' of bricks provided at one end of the tank and prefer to remain in the angle provided by the tank side and water surface when at rest. These animals are, of course, also readily distinguishable because they do not react to visual stimuli.

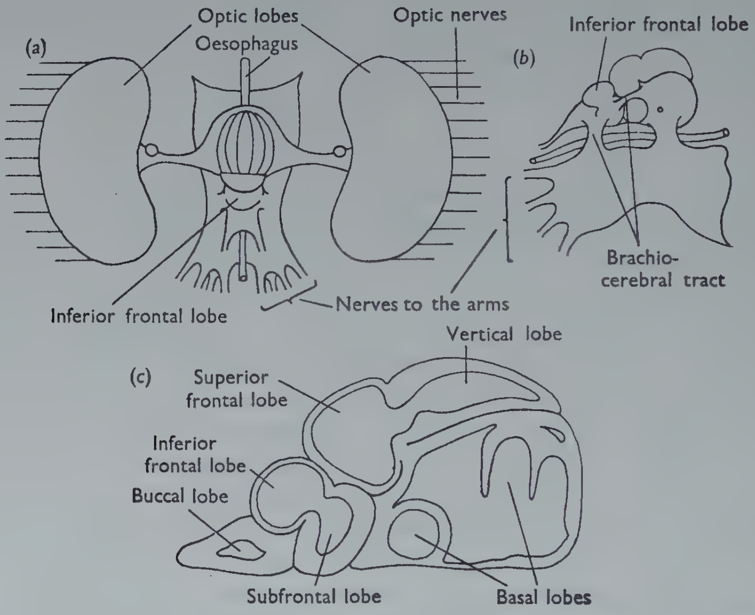

Fig. 1. Parts of the brain of *Octopus* mentioned in the text: (*a*) is a diagram of the brain, seen from above after removal of the cartilage surrounding the central mass; (*b*) is the same seen from the left side; and (*c*) is a median longitudinal section through the supra-oesophageal lobes only. (*c*) is used as the standard diagram on which lesions are plotted in Figs. 2 and 3. There is no subfrontal lobe in decapods, this being an octopod elaboration of the inferior frontal. In the text the inferior frontal and subfrontal are referred to collectively as the 'inferior frontal system'.

EXPERIMENTAL METHODS

A variety of training procedures was used. These can be classed as setting problems of two distinct orders of complexity, as follows.

A. *Simple problems*

In these experiments the animals were required to stop reacting positively in a situation normally eliciting a positive response. As a visual learning test they were trained not to attack crabs, by giving them small electric shocks when they did so. As a test of capacity for touch learning, the octopuses were repeatedly presented with a small cylindrical object and trained not to take it by giving them shocks if it was passed under the web to the mouth. Unoperated controls typically learned not to attack crabs or not to take the test object after three or four shocks. In other tactile tests no shocks were given, the animals being allowed to examine and reject the test object regardless of how long they took to do so or whether the test object was passed in towards the mouth during the course of the examination; again, control animals learned after three or four trials to reject the test object without first passing it to the mouth.

B. *More difficult problems*

Here the octopuses were required to learn to distinguish between two situations, to react positively in one and negatively in the other. As a visual test they were trained to discriminate between a rectangle shown vertically and the same rectangle shown horizontally, and as a tactile test to discriminate between two Perspex cylinders, one of which had a smooth surface and the other a surface roughened by grooves. The animals were rewarded with pieces of fish for correct responses in the 'positive' situation, and given electric shocks for errors in the 'negative'. The two discriminations are about equally difficult for *Octopus*, and in both cases controls typically made 75 % or more correct responses after the first twenty or thirty trials.

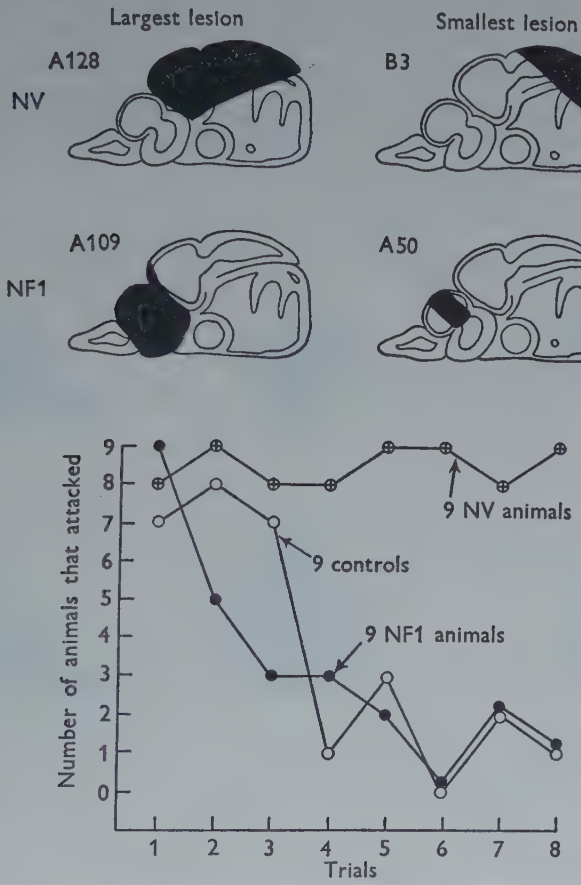

Fig. 2. A summary of the results of training octopuses not to attack crabs. Each trial lasted for 2 min. and there were four trials per day. The nine 'NV' animals with lesions to the visual learning system were the same octopuses as the controls, training being continued after the operation. 'NFI' animals had lesions in the inferior frontal system. Lesion maps show the area removed from the brains of those individuals having the largest and smallest lesions in each group. They are plotted on a standard diagram representing a median longitudinal section through the supra-oesophageal part of the brain (see Fig. 1).

RESULTS

A. *Simple problems*

(1) *Training not to attack crabs*

An untrained octopus will come out of its home of bricks and attack crabs that it sees at the other end of its tank. In the experiments a crab was shown, suspended on a line by which it was kept moving about until the octopus attacked. The crab was then snatched away at the last possible moment, and the octopus given a 6–9 V. a.c. shock through electrodes attached to the end of a probe. The shock causes the animal to retreat into its shelter and the crab can be replaced, the process being repeated until the end of the trial period. In the present series, each trial lasted for 2 min., and typical animals attacked two or three times during the first trial. There were four trials per day, not less than 1 hr. apart.

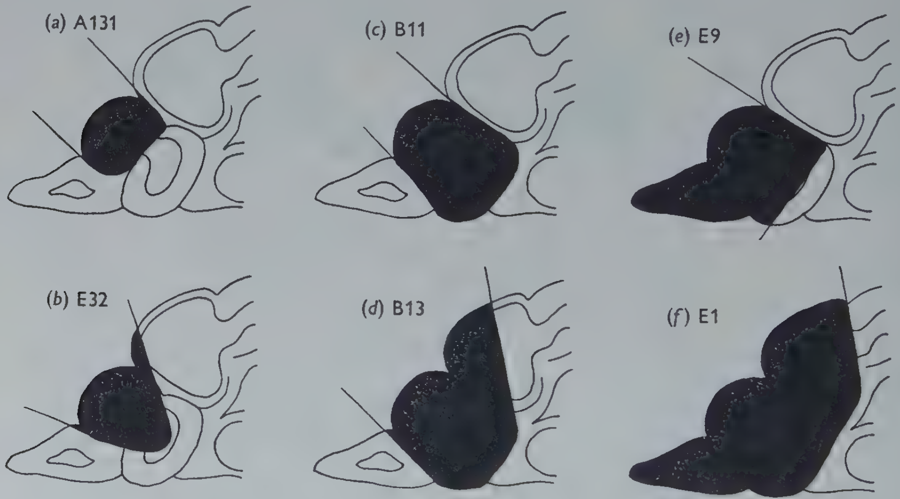

Fig. 3. Lesions made for the experiments summarized in Tables 2 to 5. Six examples are plotted here; the lesions made in the other animals are classified below according to the sample (*a* to *f*) that they most closely resembled.

A109—*c*
A112—*c*
B11—*c*
*B*24—*a*
B25—*a*
B101—*a*
B102—*c*
D35—*b*
D36—*d*
D37—*d*
E6—*e*
E8—*f*
E10—*d*
E11—*f*
E17—*b*
E34—*e*
F13—*b*
F15—*a*
F25—*e*
F28—*a*

E7 had only the buccal lobe removed.

Animals without brain lesions and animals with parts of the inferior frontal system removed learned quickly under these conditions, and very few of them attacked more than once at the second trial (Table 1). At the third trial only about half of the animals attacked at all, and after that attacks were infrequent. Most individuals will make occasional attacks on crabs even after long training not to do so. This implies that the negative training overlies rather than replaces the effects of a long previous experience of crabs as food, a matter that is discussed more fully elsewhere (Young, 1961).

The behaviour of octopuses with vertical lobe lesions under these conditions is in any case quite distinct; they seem to be unable to learn to leave crabs alone. The animals continue to attack despite shocks, and show very little, if any, improvement in performance as training continues (Table 1).

A summary of these results, with maps showing the range of lesions concerned, is given in Fig. 2. It should be noted that the animals with vertical lobe lesions were the same octopuses as the controls, being trained both before and after operation; removal of the vertical lobe abolishes all trace of previous learning as well as making it difficult or impossible to retrain the animals.

Table 1. *Results of training not to attack crabs seen at a distance*

There were four trials per day and each trial lasted for 2 min., during which the octopus was given an electric shock whenever it attacked the crab.

	Number of attacks at each trial											
	1 day				2 days				3 days			
Controls												
A123	3	3	1	3	1	0	1	1	0	0	0	1
A128	3	2	1	0	0	1	0	0	0	1	.	.
B3	2	1	1	0	2	0	0	0	0	0	0	1
B7	2	1	2	0	1	0	1	0	0	0	0	0
B15	1	1	0	0	0	0	0	0	1	1	0	0
B16	1	1	1	0	0	0	0	0	0	0	0	0
B19	0	1	2	0	0	0	0	0	0	1	0	0
B21	0	1	0	0	0	0	0	0	0	0	0	1
B23	1	0	1	0	0	0	0	0	0	0	0	0
Animals with the vertical lobe removed (The same octopuses as the controls, retrained after operation)												
A123	2	2	1	1	1	1	2	1	0	2	1	1
L A128	2	1	3	2	2	1	1	3	.	.	.	.
S B3*	2	1	1	2	1	1	1	1	1	2	2	1
B7	2	1	4	1	1	1	1	1	1	1	2	1
B15*	1	1	0	1	2	1	1	1	1	0	0	1
B16*	2	1	1	1	1	2	1	1	2	1	0	1
B19*	1	1	1	1	1	1	1	2	0	1	1	2
B21*	1	1	1	0	2	1	1	1	1	1	1	1
B23*	0	3	1	1	1	1	0	1	1	1	1	1
(A further twenty such experiments gave the same result)												
Animals with lesions in the inferior frontal system												
A46	3	0	1	0	1	0	0	0	0	0	0	0
S A50	2	1	1	0	0	0	0	0	0	0	0	0
A73	3	2	0	0	0	0	0	0	0	0	0	1
A77	3	0	0	0	0	0	1	0	0	0	1	0
A81	3	1	0	2	1	0	0	1	2	0	0	0
A83	2	0	0	0	0	0	1	0	0	1	0	0
L A109	1	1	0	1	0	0	0	0	0	0	0	0
A112	3	2	1	1	0	0	0	0	0	0	0	0
A131	1	0	0	0	0	.	.	.	.	.	.	.

S = animal with the smallest lesion in the group.
L = animal with the largest lesion.
* = Maps of the lesions in these animals have already been published in Wells & Wells, 1957*b*, where an account of their subsequent performance in a tactile discrimination is also given.

(2) *Training not to take an object touched*

(*a*) *Repeated presentations of the same object, with punishment for positive responses.* Octopuses were required to learn to reject a Perspex cylinder repeatedly presented to them. Experiments of this sort with control animals and with octopuses having vertical lobe lesions have been described elsewhere (Wells, 1959*a*, *b*); with trials at 3 or 5 min. intervals both learn quickly and after taking the object, and receiving shocks for doing so three or four times, begin to reject the cylinder by thrusting it away when it is presented. Some typical performances drawn from two longer series given in Wells (1959*a*, *b*) are shown in Table 2, where they may be compared with the results of similar experiments made on octopuses after removal of parts from the inferior frontal system.

In these experiments the behaviour of animals with inferior frontal lesions is so curious that it must be considered in some detail. At the first trial an octopus such as E9 (a map of the lesion is included in Fig. 3) behaves quite normally, grasping and passing the object under the interbrachial web just like an unoperated animal. But whereas an unoperated octopus will stop taking a test object after three or four trials, an animal with an extensive inferior frontal lesion will continue to take the object even though it receives an electric shock on every occasion. On the face of it, this indicates no learning. But an octopus with the inferior frontal system removed is evidently able to recognize some elements of the test situation, even though it performs incorrectly by taking the test object, for at nearly every trial after the first three or four, it grasps the cylinder and moves away, dragging it around the tank before passing it in towards the mouth. After a number of trials, presentation of the cylinder is regularly followed by headlong flight, but the octopus does not let go and eventually (generally as soon as it stops moving) passes the object under the interbrachial web to the mouth (Table 2). The performance of animals with lesions limited to the inferior frontal system is thus quite different from that of animals with large lesions including the vertical and basal lobes as well as the inferior frontal, for while the latter also continue to take the test object and receive shocks, they do so without any apparent change in their other behaviour (Wells, 1959*b*). Animals with the basal lobes destroyed cannot, of course, run away, because the lesions interfere with mechanisms co-ordinating movement; but they are at least potentially able to depress the head and draw away from a stimulus (Boycott & Young, 1950), and they do not. The difference between the animals which learn to run away after making contact and those that do not change their behaviour is presumably due to the integrity of the visual learning system in the former, and it is significant that the only animal from the present series which failed to learn either to reject the object or to run away had the superior frontal lobe, a part of the visual learning system, destroyed (E1, Table 2 and Fig. 3).

In most of the animals used for the experiments summarized in Table 2 the brachio-cerebral tracts were cut on both sides of the brain. It has generally been assumed that these predominantly sensory tracts are the main channel for tactile sensory input to the upper part of the brain. Yet shocks apparently have an effect upon the visual learning system whether the brachio-cerebral tracts are cut ot not, which implies a second sensory input channel, through the basal lobes or the brachio-optic

Table 2. *Results of training blinded octopuses not to take an object repeatedly presented to them*

At each trial a Perspex cylinder was presented by touching it against one of the arms; the octopus was given an electric shock if it passed the cylinder under the interbrachial web to the mouth (+ response). Controls and animals with the vertical lobe removed learn to reject the object (− response) after three or four trials (the examples given are typical performances from a series of such experiments reported in Wells, 1959*a*, *b*). Trials were at 3- or 5-min. intervals; up to twenty trials were given in a session if the octopus failed to learn, with breaks in training of at least 6 hr. between sessions.

Controls	Arm	Trials																			
B97	–	+	−	+	−	−	−	−	−	−	−	.	.	.	.	.	.	.	.	.	.
B99	–	+	+	+	−	−	−	−	−	−	−	.	.	.	.	.	.	.	.	.	.
		Animals with parts of the visual learning system removed																			
B91	–	+	+	+	+	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−
B121	–	+	+	−	−	−	−	−	−	−	−	.	.	.	.	.	.	.	.	.	.
		Animals with lesions to the inferior frontal system																			
E1	R2	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+
	L2	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+*	+	+	+	+
E6	R2	+	+*	+*	+*	+*	+*	+*	+*	−*	−*	−	−*	−*	−*	+	−*	−*	+*	−*	−
	L2	+	+*	+*	+*	+*	+*	+*	+	+	+*	+*	+	+	+	+*	+*	+*	+	+	+
E8	R2	+	+	+	+	+	+	+	+	+	+*	+*	+	−	−	+	−	−	−	+	−
E9	L2	+	+	+	+	+*	+*	+*	+	+	+	+*	+*	+*	+*	+*	+*	+*	+	+	+*
	L2	+	+	−	+*	+	+*	−*	+*	+	+*	+*	+*	+*	+	+*	+	+*	+*	+	+
E10	R2	+	−	+	+	−	+	+	−	+	−	+	+*	−	+	−*	+	−	+*	−	+
	R2	+	+	+*	+*	+*	−	+*	+	−	−*	−	−	+	−	−	−	+	+*	+*	+*
E11	R2	+	+	+	+*	+*	+*	+	−	+	+*	−*	+*	+*	+*	+*	+*	−*	+*	+*	+*
	L2	+	+	+*	+*	−*	+*	+*	+*	−*	+*	−*	−	+	+	+*	−*	−*	−	+*	+
E17†	R2	−	−	.	.	−	−	.	.	−	−	.	.	−	−	.	.	−*	−*	.	.
	L2	.	.	+	+	.	.	+	+	.	.	+*	+	.	.	+	+	.	.	+	+
	R2	.	−	.	−	.	−	.	−*	.	−*	.	−*	.	−*	.	−*	.	−*	.	−
	L2	+*	.	+*	.	+*	.	+*	.	+	.	+*	.	+*	.	+*	.	+*	.	−*	.
F34	L2	+	+	+	+	−*	+	+*	+*	+	+*	+*	−	+*	−*	+*	+	+	+*	+*	+*
	R2	+	+	+	+	+	+	+*	+*	+*	+*	+*	−*	+	+	+*	+*	−*	+*	+*	−*

* Shows that the animal ran away, trailing the object around the tank before taking or rejecting it (see text).

† In the case of E17, the right and left sides of the animal were tested alternately in the course of the twenty-trial sessions.

tract. It is not known which receptors are concerned when an octopus reacts to an electric shock.

(*b*) *Habituation experiments.* For these the same inedible cylinder was presented repeatedly, but there was no punishment for a positive response. An unblinded control animal will typically reach out to take a test object as it is dropped into the tank, and pass it at once under the interbrachial web to the mouth. After struggling to break it apart, and biting it for a minute or two (Wells & Wells, 1956), the octopus will reject the object, passing it out along the suckers and finally pushing it away with an armtip. After two or three such attempts to eat it, the cylinder is rejected when presented, without first being passed under the web to the mouth. The time taken to reject the object is somewhat variable, since it depends upon whether the octopus thrusts it away or merely removes its own arm after feeling the object over; commonly it falls from a minute or more to less than five seconds within five or six trials (Table 3).

Animals with the vertical lobe removed behave essentially as controls do in these experiments, the most notable difference being that they take approximately six times as long as controls to examine and reject the object on the occasion of its first presentation (Wells & Wells, 1957*c*). In general they are somewhat slower to learn than controls, taking the test cylinder slightly more often and retaining it on average for a little longer than controls do.

The behaviour of animals with lesions in the inferior frontal system is again quite distinct from that of controls, or of octopuses with lesions in the visual learning system. Out of the six animals tested, only one (B101) learned to reject the test object within the first twenty trials and four of the animals (A131, B11, B13 and B102) showed little or no improvement in performance, although two of them eventually ceased to react, apparently having learned to recognize the test object by sight. Considered as a whole the group with inferior frontal lesions not only passed the test object under the web to the mouth much more often than the others but also kept it there for longer on each occasion. Thus they took the test object at 67% of the trials, twice as often as controls (28%) and animals with vertical lobe lesions (34%). They retained the object for 2 min. or more on 65% of these occasions, compared with 22% by octopuses with vertical lobe lesions—controls never kept the object for as long as this after the first trial, which is not included in calculating the figures. Indeed, the difference in performance of animals with inferior frontal lesions on the one hand, and controls or animals with vertical lobe lesions on the other, is even greater than these percentages would suggest. For while controls and animals with vertical lobe lesions never retain the object for longer than a few minutes after the first trial, octopuses with lesions in the inferior frontal system quite often keep the cylinder for half an hour or more (Table 3). During these long periods, they make movements which suggest that they are struggling to wrench the cylinder apart as they might the valves of a lamellibranch. From time to time these struggles cease and the object is rejected to the edge of the web, or even part of the way along an arm, only to be retaken and the performance repeated. Indeed it appears that animals such as B11 or B13 (Table 3) only succeed in rejecting the test object at all when by chance they fumble and lose contact with it.

The poor tactile performance of these animals does not, however, seem to interfere with their learning to recognize the test object by sight. Thus tests with B11 had to be

Table 3. *The time taken (in minutes and seconds, to the nearest 5 sec.) to reject an object presented by dropping it into the tank near to the octopus.*

Tests continued until the animal no longer reached out to grasp the object (i.e. had learned to recognize it by sight), each trial beginning 2 min. after the end of the one before. Times in bold figures (**55**) show trials at which the test cylinder was passed under the interbrachial web to the mouth.

	Controls*		Vertical lobe removed†		Animals with lesions in the inferior frontal system (B99 also had the basal lobes removed)							
Trial	A121	A133	B112	B113	A131	B11	B13	B24	B25	B99	B101	B102
	s.	m. s.	m. s.	m. s.	m. s.	m. s.	m. s.	m. s.	m. s.	m. s.	m. s.	m. s.
1	55	1 50	15 15	40 35	1 5	8 0	72 0	45	180 0+	56 0	9 0	30
2	10	45	1 0	3 10	1 0	23 00	68 0	1 10	180 0+	20 35	3 0	3 00
3	5	30	5	40	1 0	10	123 0	1 40	.	1 55	10	25
4	.	25	5	1 0	1 30	3 0	41 0	3 0	.	8 0	5	2 0
5	.	10	2 5	30	1 15	15 35	31 0	40	.	6 40	5	2 30
6	.	45	5	20	5 20	2 5	17 0	2 0	.	1 30	5	2 0
7	.	25	1 5	30	10	3 50	8 0	2 30	.	13 30	10	5 0
8	.	10	5	5	5	14 30	6 0	1 0	.	5 20	5	1 30
9	.	20	1 10	5	20	13 25	7 0	4 10	.	45	5	2 30
10	.	10	15	5	15	45	12 0	1 15	.	1 20	5	1 0
11	.	15	30	5	2 0	3 40	43 0	5	.	1 10	.	20
12	.	15	10	5	25	12 0	4 0	15	.	40	.	2 20
13	.	10	5	5	3 5	6 50	13 0	7 40	.	15	.	2 0
14	.	5	5	10	1 35	9 0	.	25	.	10	.	2 0
15	.	20	5	10	5	40	.	4 10	.	2 0	.	35
16	.	5	10	40	2 25	30	.	5	.	1 20	.	40
17	.	5	5	1 5	1 45	5	.	2 50	.	10	.	40
18	.	15	20	50	2 10	.	.	1 55	.	5	.	1 30
19	.	10	5	20	10	.	.	20	.	55	.	30
20	.	.	5	30	1 25	.	.	5	.	5	.	.

* The best and the worst performances from five animals tested; details of the performance of the other three are given in Wells & Wells, 1957*c*. A121 had the optic lobes removed, and the objects were presented to this octopus (and to B99 which was also blind) by touching them against an arm.

† Details of the performance of six more octopuses with the vertical lobes removed are given in Wells & Wells, 1957*c*.

stopped after the seventeenth trial because the animal refused to reach out an arm and grasp the cylinder when it was dropped in, while a second poor performer (B24) would not take the object after the seventeenth trial unless this was made to roll about the floor of its tank by the experimenter; the cylinder was evidently recognized visually, but was, nevertheless, passed to the mouth when touched. In controls, and in animals lacking the vertical lobe, learning by touch regularly precedes learning by sight under these conditions.

B. *Performance when trained to do more difficult tasks*

(1) *Visual discrimination experiments*

A great many experiments have been made upon the capacity of octopuses to learn to distinguish between figures before and after vertical lobe removal. For a summary of these experiments see Young (1961). Visual discrimination and learning after inferior frontal and subfrontal lesions has, in contrast, scarcely been investigated. Boycott & Young (1955) cite two instances (without, however, giving any details, or maps of the lesions concerned) in which removal of the inferior frontal had no effect upon the performance of octopuses that had already been trained to distinguish between the situations 'crab' and 'crab plus white plate'; after vertical lobe removal, octopuses are unable to learn to make this particular discrimination and continue to attack in both situations.

In the present instance animals with lesions to the inferior frontal system were trained to discriminate between two 2 × 10 cm. rectangles, one being shown horizontally and the other vertically. This discrimination is quickly learned by controls, which regularly make 75% or more correct responses after the first twenty or thirty trials; octopuses with the vertical lobes removed can also be taught to make it, but require three or four times as many trials as controls to reach the same standard of performance (Young, 1961).

For the experiments summarized in Table 4 seven unoperated octopuses were trained to distinguish between horizontal and vertical rectangles; these animals together made 69% correct responses in training periods of up to fifty-six trials. Four of them then had parts of the inferior frontal system removed; they continued to discriminate after the operation, the best animal (E32) making 70% correct post-operational responses. The other three (E7, E34 and F25) had the buccal lobe damaged or removed in the operation and were subsequently unable to eat their rewards; these octopuses nevertheless showed that they were still able to discriminate, together attacking the positive shape sixteen times and the negative only five times before ceasing to attack altogether. Three further octopuses (F13, F15 and F28) were operated upon without pre-training. These were all able to eat their rewards post-operationally and together totalled 74% correct responses over the whole period of training; in the last forty training trials these animals made 88% correct responses. Details of all these experiments are given in Table 4.

Clearly removal of large parts from the inferior frontal system does not prevent visual learning, nor does it abolish memories of learned visual discriminations. Indeed, the performance of the four animals that were still able to eat post-operationally (E32, F13, F15 and F28 together made 74% correct responses in training—84% in the last

Table 4. A summary of performance in learning to discriminate between horizontal (H) and vertical (V) rectangles *after removal of parts from the inferior frontal system*

Some of the animals were also trained before operation ('Op' in the table); in these instances the lesion was made in the evening at the end of a day's training, and tests resumed next morning. There were eight trials per day (4+, 4−) at intervals of not less than 1 hr. The table shows how many attacks were made on each of the rectangles, and the total errors (column E) for each day.

Controls								
E27			E28			E30		
H+	V−		H+	V−		H+	V−	
+	−	E	+	−	E	+	−	E
2	1	3	2	2	4	2	1	3
3	1	2	0	0	4	2	1	3
2	0	2	2	2	4	4	1	1
2	0	2	1	1	4	3	0	1
3	2	3	3	1	2	4	1	0
4	2	2	4	1	1	4	0	0
71‡			61‡			84‡		

Animals with inferior frontal lesions†																				
E7			E32			E34			F13			F15			F25			F28		
H+	V−		H+	V−		H+	V−		V+	H−		V+	H−		V+	H−		H+	V−	
+	−	E	+	−	E	+	−	E	+	−	E	+	−	E	+	−	E	+	−	E
2	2	4	1	2	5	2	1	3	Op.			Op.			3	1	2	Op.		
0	0	4	3	0	1	2	1	3	2	1	3	4	2	2	4	2	2	4	4	4
3	0	1	4	0	0	1	4	7	1	0	3	0	0	4	4	1	1	1	2	5
3	1	2	3	1	2	0	2	6	0	0	4	2	0	2	Op.			2	1	3
2	1	3	3	1	2	1	0	3	1	0	3	4	0	0	1	0	3	2	3	5
3	1	2	Op.			3	1	2	1	0	3	4	0	0	3	2	3	4	2	2
Op.			1	1	4	4	1	1	3	0	1	4	2	2	1	2	5	1	3	6
0	0	4	3	1	2	Op.			0	0	4	4	0	0	1	0	3	3	1	2
0	0	4	1	0	3	1	0	3	1	0	3	4	1	1	1	1	4	3	0	1
1	0	3	3	0	1	3	0	1	1	0	3	3	1	2	0	0	4	4	2	2
1	0	3	2	0	2	0	0	4	1	0	3	4	0	0	0	0	4	4	0	0
1	0	3	75‡ 70§			55‡ 66§			1	0	3	4	0	0	79‡ 54§			3	1	2
0	0	4							0	0	4	4	0	0				4	1	1
1	0	3							3	0	1	4	1	1				4	1	1
1	0	3							1	0	3	3	0	1				68§		
0	0	4							0	0	4	2	0	2						
66‡ 57§									1	0	3	86§								
									1	1	4									
									3	0	1									
									3	0	1									
									4	0	0									
									3	0	1									
									4	0	0									
									4	1	1									
									2	0	2									
									70§											

† Maps of lesions are included in Fig. 3.

‡ Percentage correct responses pre-operationally.

§ Percentage correct responses post-operationally.

Table 5. *A summary of the results of tactile training experiments made after removal of parts from the inferior frontal system*

The animals were trained to distinguish between two Perspex cylinders, P 1 (which had grooves cut into it) and P 4 (a smooth cylinder) or P 3 (another grooved cylinder, used in two experiments only). The table shows the number of times that each object was taken, and the total errors (column E) for each day, or each session of twenty trials (D series only).

Experiments in which the octopuses were trained at 8 trials (4+, 4−) per day, at hourly intervals												Experiments in which the animals were trained at 40 trials per day in two groups of 20 (10+, 10−), 6 hr. apart									Experiments in which there were 4 groups of trials (each 1+, 2−) per day, 2 hr. apart								
B 11			B 13			B 24			B 25			D 35			D 36			D 37			A 109			A 112			A 112		
P4+/P1−			P4+/P1−			P1+/P4−			P1+/P4−			P1+/P4−			P4+/P1−			P4+/P1−			P1+/P3−			P3+/P1−			P4+/P1−		
+	−	E	+	−	E	+	−	E	+	−	E	+	−	E	+	−	E	+	−	E	+	−	E	+	−	E	+	−	E
1	3	6	2	4	6	4	4	4	4	4	4	4	7	13	3	2	9	1	1	10	3	7	8	3	4	5	4	7	7
0	3	7	0	2	6	3	4	5	4	4	4	3	2	9	0	0	10	0	0	10	0	5	9	4	6	6	3	7	8
1	1	4	2	3	5	4	4	4	4	4	4	3	2	9	1	0	9	3	0	7	2	5	7	3	7	8	3	4	5
3	4	5	0	2	6	4	4	4	4	3	3	2	0	8	1	0	9	2	0	8	2	3	5	3	6	7	2	5	7
1	3	6	1	3	6	4	4	4	4	3	3	4	2	8	0	0	10	0	0	10	0	4	8	2	3	5	2	7	9
1	0	3	2	3	5	4	4	4	4	4	4	1	1	10	2	0	8	1	0	9		38*			48*			40*	
0	0	4	4	4	4	4	4	4	4	4	4	1	0	9	0	0	10	0	0	10									
1	3	6	4	3	3	4	4	4	4	4	4	0	0	10	1	0	9		54*										
3	1	2	2	1	3	4	4	4	4	3	3	2	2	10		54*													
2	1	3	3	4	5	4	4	4	4	4	4	0	0	10															
0	1	5	3	2	3	4	4	4	4	3	3		52*																
3	1	2	2	4	6	3	4	5	4	4	4																		
4	2	2	3	2	3	2	2	4	4	3	3																		
1	3	6	2	4	6	3	3	4	4	4	4																		
3	4	5	3	4	5	3	2	3	4	3	3																		
0	2	6	3	3	4		50*			55*																			
	43*			42*																									

* Percentage correct responses (there was no pre-operational training).

forty trials) strongly suggests that inferior frontal removal is entirely without effect upon the capacity to learn to discriminate by sight. What the operation does sometimes do is alter the chances of the animal attacking at all. Thus F13, which discriminated well, attacking the positive shape forty-one times and the negative only three times in ninety-six trials with each, nevertheless scored only 70% correct responses because of the very large number of failures to attack in the positive situation (Table 4). A possible explanation of this post-operational reluctance to attack, which was also apparent in the case of the four octopuses trained before operation, is dealt with in the discussion.

(2) *Tactile discrimination experiments*

Blinded octopuses can readily be trained to discriminate between grooved and smooth Perspex cylinders, controls regularly making 75% or more correct responses the end of the first twenty or thirty trials (Wells & Wells, 1957*a*, *b*). If the vertical lobe is removed learning is slowed or prevented. The animals tend to take any object that is presented and most of them fail to learn to discriminate when training is at a rate of eight trials (4+, 4−) per day. Increasing the rate of training to forty trials per day, in two sessions of twenty trials (each 10+, 10−), however, depresses the overriding tendency to react positively and under these conditions it becomes possible to teach octopuses lacking the vertical lobe, the animals requiring only about 50% more trials than controls to attain standards of 75 or 85% correct responses (Wells & Wells, 1957*b*). Once the vertical lobe has been removed, the rest of the supra-oesophageal brain can be taken out without further effect upon touch learning, provided that the inferior frontal system and the buccal lobe are left intact (Wells, 1959*b*).

Octopuses with extensive inferior frontal lesions do not learn to discriminate by touch, whatever the rate of training. With infrequent trials (eight per day) some of the animals (B24 and B25 in Table 5) take almost every object that is presented and in this resemble animals with vertical lobe lesions; an increase in the rate of training to forty trials per day is, however, followed by a progressive *indiscriminate* decline in the proportion of objects that is taken (D35, D36, D37—Table 5), so that there is no decrease in the number of errors made.

DISCUSSION

(1) *Errors after lesions to the inferior frontal system*

A possible explanation of the poor performance of operated animals in repeated presentation experiments is that damage to the inferior frontal system prevents octopuses from letting go. This would be compatible with von Uexküll's (1895) observation, using *Eledone*, that a transverse vertical cut through the middle of the supra-oesophageal brain produces octopuses that have difficulty in releasing things that they have grasped. Such a cut would damage or disconnect the interior frontal from the rest of the supra-oesophageal brain. Von Uexküll postulated separate centres for prehension and release of the suckers, separated by the cut, but it has not subsequently been possible to trace these centres and this is probably the wrong explanation of his results (see below, and Boycott, 1961). There is certainly, however, very often a notable 'stickiness' about the suckers of animals with inferior frontal lesions, though the effect is curiously irregular. It is evident when the animals are touched and allowed to grasp the hand or a probe; having done so they commonly seem unable to let go, though apparently making every effort to escape by discharging jets of water and ink. In contrast there is no trace of interference with normal locomotion as the animals wander about their tanks. They do not remain stuck to surfaces unless an attempt is made to pick them up or to pull objects away from them.

These actions are explicable in terms of a dual control of arm movements, for which there is already some evidence. Thus the arms are known to be controlled partly by centres in the basal lobes, without which there is no co-ordination of their movements, no walking and no swimming (Boycott & Young, 1950). Presumably, there is a proprioceptive sensory input to these centres. Superimposed on this mechanism for the regulation of locomotion is a second system, acting almost independently on a basis of surface tactile information and normally concerned in taking or rejecting potential food objects. Isolated arms can be stimulated by touch or taste to perform all of the movements used in grasping and passing small objects to the mouth. Whether they do so or not is regulated by the inferior frontal system, for with this intact octopuses still perform satisfactorily in tactile discrimination experiments even when all of the other supra-oesophageal lobes have been removed. Without it they normally take all objects presented, despite the fact that they are potentially able to make rejection movements, which also seem to be regulated at the arms level (Wells, 1959*b*). Even in intact octopuses the decision whether to take or reject an object touched is made entirely on a basis of surface tactile information, for octopuses cannot be taught to make tactile discriminations that would require their taking proprioceptive information into account (Wells & Wells, 1957*a*; Wells, 1961).

In the present series of experiments octopuses had the inferior frontal system damaged or destroyed, with the motor control regions of the basal lobes left intact. Thus in repeated presentation experiments they could run away from a contact, but had no means of inhibiting the arm reflexes that cause them to pass the test objects in towards the mouth.

A similar failure to inhibit reflex grasping by the suckers may well be responsible for the failure to attack in some visual experiments and for the 'stickiness' of animals with inferior frontal lesions when picked up. In these situations there must be a sudden increase in activity within the arm nerve cord as the creature prepares to rush forward or away, and in the absence of any higher control from the inferior frontal system this would perhaps incidentally enhance the activity of the reflexes controlling prehension, making it difficult or impossible for the animals to let go.

(2) *Visual and tactile learning centres*

The experiments described above show that visual and tactile learning are carried out in different parts of the brain of *Octopus*. The optic lobes seem to be exclusively concerned with visual learning and the inferior frontal and subfrontal with learning to recognize situations by touch. The vertical lobe alone plays some part in both tactile and visual learning. If it is removed, animals that have learned to make discriminations before the operation show little trace of their training, and they can only be retaught with difficulty. The immediate effect of removal suggests that it functions at least partly as a store; but it may also serve in establishing memory traces elsewhere in the brain (for discussions of the probable functions of the vertical lobe see Young, 1961; Wells, 1962).

Separation of so much of the mechanism concerned in learning into two distinct regions would seem, on the face of it, to be a curious way of organizing any nervous system. But it could have arisen because the ancestors of the present-day octopods were pelagic and predominantly dependent upon vision. We know at least that cretaceous octopods had both fins and an axial skeleton, like modern decapods (Roger, 1944) and that, in modern decapods, the inferior frontal system is poorly developed (there is, for example, no subfrontal lobe (Wirz, 1959)). Presumably such animals depend on touch only in as much as it signals the success or failure of actions that have been set in motion by visual stimuli, and it may well be that as the octopods took to the bottom in cretaceous times the visual system was already too specialized for the analysis of visual stimuli to be useful as an extractor of the relevant information from an increasingly complex tactile input.

Thus the results of visual discrimination experiments (Sutherland, 1960) and the structure of the optic lobes (Young, 1960) both suggest that the mechanism in the brain of *Octopus* for analysing visual stimuli is organized to extract ratios of extents from patterns of stimulation of the retina (Young, 1961). A mechanism capable of filtering inputs in this way might be ill-adapted for dealing with tactile information where the spatial patterning of the neurones that are firing is of secondary importance to variations in their rate of firing. Octopuses distinguish objects that they touch by their texture and taste, not by their shape (Wells & Wells, 1957*a*). A separate mechanism for tactile learning may have developed in the inferior frontal lobe, not so much because there is any advantage in separating tactile and visual information stores, as because of an incompatability in the way that visual and tactile inputs must be coded for storing. The stimuli affecting the receptors are in both instances patterned in distribution and intensity. But whereas in the optic lobes the extraction process preserves as relevant the spatial distribution of the stimulus pattern, in the arms and inferior frontal system it is the degree of stimulation that is the more important element and its spatial distribution is discarded by the filter.

So far we know very little about the anatomy of the inferior frontal system. All that can be said at present is that there is little trace of the elaborate arrangements reported by Young (1960) for the optic lobes. The inferior frontal and subfrontal, on the other hand, do have a good deal of structural similarity to the superior frontal and vertical lobes. This, and the fact that the vertical lobe acts as a store both for tactile and for visual memories, could mean that the arrangements found in the optic lobes serve to convert patterned visual information into a form similar to that in which tactile information is stored, that is, perhaps, into trains of nerve impulses with frequencies characteristic of particular patterns of stimulation. In this situation storage might be envisaged as a property of groups of cells, so interconnected as to discharge collectively only in response to a limited range of input frequencies, rather than by 'typing' of cells responding to particular spatial patterns of input, as has been suggested by Young (1961).

SUMMARY

1. Octopuses were trained to stop reacting to visual and tactile stimuli normally eliciting positive responses, and to make visual and tactile discriminations. The effect on performance of removal of parts from the brain was observed.

2. It appears that *Octopus* has two learning systems, one in the inferior frontal and subfrontal lobes, dealing with tactile discrimination on a basis of the proportion of sense organs excited, the other in the optic lobes, handling visual discrimination on a basis of the pattern of sense organs excited.

3. The vertical lobe plays a part in learning by either system, and is to some extent a store for both tactile and visual memories.

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RETINAL ORIENTATION AND THE DISCRIMINATION OF POLARIZED LIGHT BY OCTOPUSES

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(*Received* 30 *September* 1961)

INTRODUCTION

Moody & Parriss (1961) showed that *Octopus* was capable of discriminating between two sources of plane polarized light, whose planes of polarization were at right angles. These experiments did not entirely eliminate the possibility of a discrimination based on differential reflexion. The experiments described below confirm that *Octopus* can distinguish between sources emitting light polarized in different planes and show that discrimination is maintained under conditions in which reflexions from the walls or contents of the aquaria or from the water surface are made to vary unsystematically.

METHOD

The slit pupil of the eye of *Octopus* normally remains horizontal, indicating that the retina remains constantly oriented with respect to gravity, whatever the position of the rest of the animal. Wells (1960) showed that if both statocysts are removed this regular orientation is lost. After the operation pre-trained animals continued to discriminate between black and white objects but were unable to distinguish between rectangles differing only in orientation. If, however, the rectangles were shown with the axes formerly parallel to the horizontal now parallel to the pupil, the discrimination continued.

If *Octopus* was basing its discrimination of polarized light sources on internal reflexions, then discrimination would only continue as long as the plane of polarization was oriented in a particular way to the fixed reflecting surfaces. By removing the statocysts and matching the electric vector to the (now irregular) orientation of the pupil, the plane of polarization is made to take up a random series of orientations with respect to the fixed reflecting surfaces. If discrimination continues under these conditions, it can be concluded that the discrimination is *not* based on differential internal reflexion.

MATERIALS AND TRAINING PROCEDURE

Small *Octopus vulgaris* Lamarck, weighing between 15 and 30 g., were obtained from the Bay of Naples and tested during July and August 1961. They were kept singly in wooden aquaria measuring 40 × 20 × 15 cm., set up as described by Wells (1960).

A torch emitting plane polarized light was constructed in the following manner. A 6 V. torch bulb was embedded in paraffin wax in a glass tube 4 cm. long and 1·5 cm. in diameter. Leads to the bulb passed out through a rubber bung in the rear end of the tube. In front of this light source were arranged a further 1 cm. of paraffin wax, and a disk of Polaroid plastic, 1·5 cm. diameter. The whole assembly was placed inside a clear glass specimen tube 5·2 cm. long and 1·75 cm. outside diameter. The inside of this tube was lined with opaque black paper. The resultant torch is shown in Fig. 1. In front view it presents a 1·5 cm. disk of illuminated polaroid, surrounded by a 1 mm. ring of non-polarized light, transmitted through the glass walls of the specimen tube. The torch was held on the end of a probe by concentric rings of wire round the outer tube. This type of construction has the following advantages:

(*a*) Its total size is small relative to the area of polaroid it presents.

(*b*) The polaroid disk is loose relative to both the wax and the overlying glass, so that slight irregularities in these two surfaces are altered unsystematically in successive presentations. This avoids a false discrimination based upon these cues.

There are no external markings indicating the vector position. This was set before each test by examining the torch through another piece of polaroid. At intervals throughout the testing period the polaroid and the diffusing wax were replaced by new material.

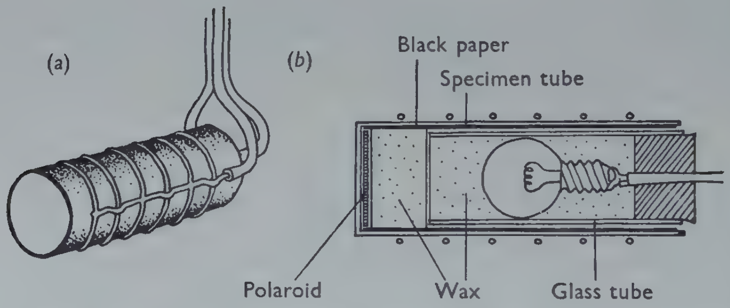

Fig. 1. The torch used in the experiments. (*a*) Shows the wires through which shocks were given and (*b*) is a section, showing internal construction. The external specimen tube, with the Polaroid sheet held in place by a wax plug, could be rotated around the internal tube containing the light source.

During training, the torch was placed in the aquarium as far as possible from the octopus, and was moved slowly up and down and from side to side for 30 sec., or until the octopus attacked it. Throughout the series the positive object was the torch with the electric vector normal to the pupil, and the negative object was the torch with the electric vector parallel to the pupil. Attacks on positive objects were rewarded with a piece of prawn, attacks on negative objects punished by a 6 V. a.c. shock given through the wire rings around the torch.

After removal of the statocysts, some modifications were necessary. Since the orientation of the retina is liable to alter as soon as the animal moves its head, the torch was presented near enough for the octopus to reach out and touch it with one arm (see Wells, 1960). Further, some of the animals had learned by this time to avoid the wire rings when touching the torch, and the punishing shock was therefore administered through a probe.

Before testing began the animals were pre-trained for 2 days, at eight trials per day, on the positive object only, and all reached 100% attack level on the second day. During testing, the positive and negative objects were each presented four times a day in the following sequence. First day: + − + − + + − −; second day: − + − + − + + −, and so on.

RESULTS

Ten animals were trained; one of these escaped and died on day 8. The other individuals made from 16 to 35 attacks out of a possible 80 during the 10 days of pre-operational training. The complete data for these animals are given in the first part of Table 1.

Table 1. *Numbers of attacks made in the positive (electric vector normal to pupil) and negative (electric vector parallel to pupil) situations before and after removal of the statocysts*

	Animal																				Total	
	A		C		G		H		K		L		M		O		P		Q			
Day	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−
1	3	3	2	2	3	2	4	3	1	2	1	2	4	2	3	3	3	2	4	2	28	23
2	2	1	2	0	0	1	3	2	3	2	2	1	1	1	3	2	3	1	4	2	23	13
3	3	1	1	1	2	1	3	2	2	0	2	0	2	1	2	1	2	1	3	2	22	10
4	3	1	1	1	0	0	4	1	2	0	2	1	1	2	3	1	1	1	3	1	20	9
5	1	1	0	0	1	0	2	1	2	2	1	1	1	1	1	0	2	1	2	0	13	7
6	1	1	2	0	3	0	3	1	1	1	2	2	2	2	2	2	3	2	2	0	21	11
7	0	0	0	1	2	0	3	1	0	0	4	0	2	0	1	1	1	0	4	0	17	3
8	0	0	1	0	1	0	2	1	2	0	4	2	3	1	3	0	1	0	1	3	18	7
9	0	0	1	1	2	0	3	2	0	0	2	0	4	2	4	2	.		3	1	19	8
10	0	0	0	0	1	0	0	0	2	1	3	1	3	0	4	0	.		4	1	17	3
Total +	13		10		15		27		15		23		23		26		16		30		198	.
Total −	8		6		4		14		8		10		12		12		8		12		.	94
11	Operation—both statocysts removed																					
12	3	1	2	2	4	1	0	1	1	0	0	0	2	1	4	1	.		.		16	7
13	3	2	3	1	1	1	3	0	1	1	2	0	2	1	1	4	.		.		16	10
14	0	2	2	0	2	0	4	1	2	2	1	0	2	0	2	1	.		.		15	6
15	2	0	0	1	3	0	0	1	3	2	.		3	1	3	1	.		.		14	6
16	1	1	1	0	4	1	2	1	1	2	.		1	2	1	1	.		.		11	8
17	0	1	1	1	2	0	2	1	2	1	.		1	2	2	0	.		.		10	5
18	1	0	0	0	0	0	.		0	0	.		4	2	4	0	.		.		9	2
19	2	0	1	0	1	1	.		3	2	.		2	1	1	0	.		.		10	4
20	1	1	1	1	2	2	.		1	1	.		1	1	1	1	.		.		7	7
21	1	1	2	1	2	1	.		2	2	.		0	0	4	1	.		.		11	6
Total +	14		13		21		11		16		3		18		23		.		.		119	.
Total −	9		7		7		4		13		0		11		10		.		.		.	61

On day 11, eight of the nine surviving animals were anaesthetized with urethane, and the statocysts removed. The experiment with the remaining animal (Q in table 1) was begun after the others and there was no time left for post-operational training. On day 12 training was resumed, this time with the electric vector matched to the now randomly oriented pupil. During this period two animals were killed by an overnight breakdown in the seawater circulation. The remaining six were trained for 10 days. The data for all the animals are given in the second half of Table 1; individuals trained for the full 10 days made from twenty to thirty-three attacks, so that the level of response was about the same as in pre-operational training.

From Table 1 it can be seen that the total numbers of attacks on positive and negative objects respectively were 198 and 94 before operation, and 119 and 61 afterwards;

in both cases the difference is significant (two-cell table of χ^2 for equal expected frequencies, $P < 0·01$).

Probably it would be safe to conclude from this alone that:

(*a*) The animals discriminated between the positive and negative objects, and

(*b*) That they continued to do so after statocyst removal.

But more accurate information can be obtained in the following way.

Table 2 and Fig. 2 show the average number of attacks made on the positive and on the negative object for each day of the testing period. Table 2 also shows the ratio (R) between these averages for each day. In it the average values of $\bar{R}$ (R) are calculated for the pre-operational and post-operational periods, being 2·81 ± 0·55 and 2·25 ± 0·33, respectively. As these values are significantly (0·001) above unity, it is confirmed that the animals distinguish between the positive and negative objects;

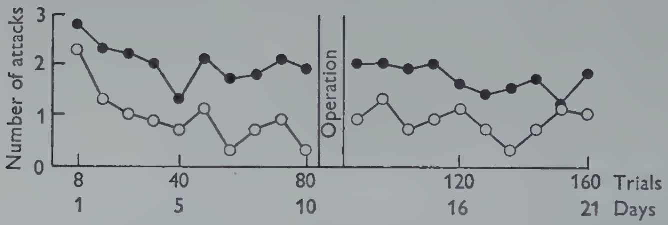

Fig. 2. The effect of statocyst removal on performance in a polarized light discrimination. ● shows the number of attacks in the +ve situation (electric vector normal to the pupil) and ○ the number of attacks in the −ve (vector parallel with the pupil) in each day of 8 trials (4+, 4−). Averages from ten animals, which had 2 days (16 trials) of pre-training to attack in the positive situation before the start of the experiment. On day 11 both statocysts were removed.

Table 2. *Average number of attacks per day in the positive and negative situations* ($\bar{x}$+ *and* $\bar{x}$−), *and the ratio* (R) *of the positive to the negative average*

Day	$\bar{x}$+ av. no. + attacks	$\bar{x}$− av. no. − attacks	R ($\bar{x}+/\bar{x}-$)	
1	2·8	2·3	1·22	Pre-operation: $\bar{R}$ = 2·81 ± 0·55
2	2·3	1·3	1·77	
3	2·2	1·0	2·20	
4	2·0	0·9	2·22	
5	1·3	0·7	1·86	
6	2·1	1·1	1·91	
7	1·7	0·3	5·67	
8	1·8	0·7	2·57	
9	2·1	0·9	2·33	
10	1·9	0·3	6·33	
11		Operation		
12	2·0	0·9	2·22	Post-operation: $\bar{R}$ = 2·25 ± 0·33
13	2·0	1·3	1·54	
14	1·9	0·7	2·71	
15	2·0	0·9	2·22	
16	1·6	1·1	1·46	
17	1·4	0·7	2·00	
18	1·5	0·3	5·00	
19	1·7	0·7	2·43	
20	1·2	1·1	1·10	
21	1·8	1·0	1·80	

and as they do not differ significantly from each other it is confirmed that the octopuses continue to discriminate just as well after removal of the statocysts as they did before.

The initial prejudice in favour of the positive object on day 1 is presumably a result of the pre-training on the positive object, and implies that the animals are immediately aware of a difference between the positive and negative objects. The discrimination improves up to the third day, which is reflected in the rise in R over this period.

CONCLUSIONS

The results obtained above confirm that *Octopus* distinguishes between sources emitting plane polarized light in planes at right angles to each other, and show that this effect is independent of extra-ocular reflexion. There remain other theoretical possibilities of extra-ocular discrimination, but these have been examined by Moody & Parriss (1961), and have been shown to be exceedingly unlikely. It is therefore concluded that the mechanism of discrimination of polarized light is intra-ocular, as suggested by these authors.

SUMMARY

1. The object of the investigation was to show that the ability of *Octopus* to detect the plane of polarization of light does not depend upon differential reflexion from its surroundings.

2. Animals were trained to distinguish between light plane polarized in the vertical plane (normal to the iris slit of the intact animal) and in the horizontal plane (parallel to the slit).

3. The statocysts were then removed, abolishing the normally fixed orientation of the eyes with respect to gravity.

4. After the operation the plane of polarization was set at each trial so as to be either normal or parallel to the iris slit. Because the eyes were now disoriented, there was no longer any constant relation between these planes and the fixed reflecting surfaces of the aquarium.

5. The animals, nevertheless, continued to discriminate correctly. It is therefore concluded that detection of the plane of polarization depends upon some intra-ocular mechanism.

The authors would like to thank the director and staff of the Naples Zoological Station for their hospitality during this investigation, and Dr K. E. Machin for his advice on the statistical treatment.

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THE CELLS FOUNDING AGGREGATION CENTRES IN THE SLIME MOULD *POLYSPHONDYLIUM VIOLACEUM*

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In an ordinary agar-plate culture of the collective amoeba, or cellular slime mould, *Polysphondylium violaceum*, the solitary phase ends when compact and increasingly refractive cell clumps develop among the unoriented cells and secrete the chemotactic factor *acrasin*. Neighbouring cells may crawl towards them, either singly or aggregated in *streams*, which relay chemotactic and contact guidance centrifugally (Shaffer, 1957*a*, 1958). As already briefly reported (Shaffer, 1959), aggregation *centres* may be started by single, differentiated cells; these have been called *founders*.

METHODS

'Ordinary' cultures were plates of 2% Difco agar, phosphate-buffered at pH 6·5 and without added nutrient, that were simply spread with a layer of *Escherichia coli* and inoculated at one side with *Polysphondylium* spores.

In 'sandwich' cultures, which were of two kinds, the cells lay between the agar and glass. Sandwiching, first employed for quite a different reason in the acrasin test (Shaffer, 1953), had advantages for microscopy in that the aggregates, as well as the individual cells, were flatter and did not project into a non-aqueous phase. Consequently, one could see, for example, all the cells within a stream, if it was not too big (Shaffer, 1958), and the phase-contrast image was greatly improved. Because development was often highly aberrant when the agar layer was itself sandwiched between two pieces of glass, an air layer was interposed. These cultures therefore differed from those recently used by several other workers for phase microscopy of slime moulds.

The 'cord' plate was designed for studying large populations at low magnification. The bottom of an optically flat Petri dish (Anumbra) served as the top of the culture chamber. It stood inside the inverted lid, the flat surfaces being kept apart by a peripheral ring of cord or plastic belting, about 4 mm. thick. A thin agar plate was spread with a layer of *E. coli* and inoculated in the centre with *Polysphondylium* spores; the agar was then transferred to the underside of the top of the sterilized culture chamber, inoculum towards the glass. The bottom of the chamber was covered by a film of water containing a trace of detergent and the cord was kept wet. Petri-dish cultures assembled in this way could be examined with the Zeiss phase-contrast microscope fitted with a long-working distance condenser.

For work at higher magnifications, agar disks of about two-thirds the diameter of the coverslips to be used were cut from very thin non-nutrient agar plates previously spread very sparsely with bacteria. Each disk was inoculated at its centre with a small number of spores, and placed, inoculum down, on a coverslip. Alternatively, the spores were deposited directly on the coverslip and left a minute to dry; this minimized their dispersal when the agar disk was applied to the glass. The coverslip was then waxed on to a glass, aluminium, or Perspex spacer; this had previously been waxed to a glass slide or another coverslip, which had then been smeared with a drop of dilute detergent to prevent the formation of water droplets. Before the long-working-distance condenser was available, and for oil-immersion work thereafter, the preparations had a total thickness of 1 mm., including both coverslips, agar layer (0·1–0·2 mm.) and air space.

EFFECT OF CULTURE CONDITIONS

Though the sandwich cultures were better for microscopy, it was possible that the agar overlay influenced development. Some parallel observations were therefore made on ordinary plate cultures. In these, at low or medium population densities, the centre of an aggregation soon became hemispherical; and its streams, if and when they appeared, grew outwards from it as what were virtually continuous lines of cells, though they were liable to break up secondarily (Shaffer, 1958). Whatever their length, the streams ended abruptly at the periphery: beyond them, there was a zone in which the separate cells, as they crowded inwards, reached a higher density than in the unaffected areas further out; but there was little tendency to form short lengths of stream out of contact with the main system. This tendency was often somewhat enhanced in sandwich cultures: in many cases, the cells at some distance from a centre lengthened and oriented soon after it became active, fell into line behind one another even before they were touching, and then linked up in short chains, which gradually joined together to yield continuous streams but initially had gaps of perhaps 100–200 μ between them. This 'stippling' of young streams was never anything like so extensive as that shown by *Dictyostelium discoideum* on ordinary plates: the zone of stippling rarely extended more than ~ 0·5 mm.

When ordinary cultures were transferred from darkness to light, there was commonly a great increase in the rate at which centres formed. Usually this began about ½–1 hr. later, though sometimes after only 20 min., and it lasted about 3–5 hr. Subsequently, there were further outbursts even in constant illumination, and these presumably resulted, as in *D. discoideum* (Shaffer, 1958), from the initial synchronization of activity. In cultures in which all the cells were ready to aggregate the size aggregations reached was determined, except at very low densities, by mutual competition, and therefore by the rate at which centres appeared in a given area. It was for this reason that the streams reached much greater lengths in darkness than in light, as they also did (Raper, 1940) in *D. discoideum*. Another factor affecting size was that a centre became much less attractive after a few hours; and if by then it had not acquired streams, it largely or completely stopped growing, even if many separate cells remained in its vicinity.

In sandwich cultures, the formation of centres was considerably inhibited. First, the outburst provoked by light was somewhat delayed, starting occasionally 1 hr, but usually 2–4 hr, after exposure. But much more strikingly, if coverslip cultures were kept in continuous darkness except for the illumination needed for periodic microscopic examination, no centres at all appeared in the 4–5 days before the cells reached the edge of the agar disk; at a density of about 50 cells/mm.^{2}, there were merely a few, minute, temporary clumps. It was possible, however, that very rare, small centres *had* formed, disintegrated spontaneously (Shaffer 1957*b*) soon afterwards, and thus escaped detection. Actually, if coverslip cultures were illuminated and then returned to darkness, although any aggregations that had appeared might continue to grow for a few hours, they all disintegrated by the following day. In many cases there still remained clouds of small, mainly separate, unelongated cells, the residual clumps in them of up to about half-a-dozen cells diminishing in size and number as dispersion continued. When such cultures were re-illuminated new centres appeared almost exclusively in sparser areas containing previously unaggregated cells, though the cloud cells re-aggregated towards them. Even if the old aggregates were re-illuminated while still compact, they acquired few if any fresh streams. In contrast, old streamless centres in ordinary cultures did sometimes attract some more cells during the next outburst of aggregation, if this occurred soon enough, though these newcomers might arrive too late to enter the initial fruiting bodies (Shaffer, 1958).

Inhibition was further examined in cord plates yielding densities of up to more than 10^4 cells/mm.^{2}. Not only could these show to what extent inhibition could be overcome by intercellular contact, there was also virtually no danger of failing to detect a centre: at medium to high densities, once one was established, aggregation would readily spread through a plate even in darkness; and although the original pattern changed considerably because parts disintegrated and the cells could re-aggregate, and because streams fragmented and could rejoin or form rings (Shaffer, 1957*b*), many cells remained aggregated for several days. Cells waiting in darkness for centres to appear became slightly more adhesive and thus tended to clump. This tendency became more conspicuous at higher densities, until when the cells covered about half the surface of the agar, almost all of them gathered into flat islands of complicated but smooth outline. At a still higher density, the cells formed a continuous, uniform monolayer, interrupted only by small lacunae, and thus the resemblance to true aggregation was less. Even with some further increase in density, the cells remained in a single layer, though compelled to thicken and to reduce their apparent size. At all densities, however, the cells were essentially isodiametric in the plane of the sandwich until a centre became active; and then oriented elongation spread rapidly outwards from it, transforming islands or splitting a continuous sheet into true streams. In darkness, aggregation was in all cases delayed upwards of a day; and provided that no cells reached the edge of the sandwich and there were no trapped, permanent, air bubbles, it did not occur in most of the lower-density plates and in a few of the highest-density ones within 5 days of cells reaching the pre-aggregation stage. Because a single centre in a high-density plate was sufficient to induce aggregation to spread through it, it seems unlikely that, cell for cell, inhibition was much reduced by increasing the density although no precise measurements were made. If illuminated at any time during the protracted pre-aggregation period even the sparsest cultures became covered in about 2–3 hr. with a rash of centres, at roughly millimetre intervals, each with an apron or radially oriented cells surrounding a multilayered core of compact cells that were approximately isodiametric in *all* directions. If centres failed to appear in a plate kept in darkness, the cells eventually lost their increased adhesiveness, and the clumps dispersed.

Sandwich cultures also strikingly revealed the remarkable change in size which the cells could undergo (Pl. 1*a*). They were, of course, the same size as the spores when they hatched, but if given nutrient they soon grew very considerably. As the cultures aged all the separate starving cells in them, whether they had ever aggregated or not, shrank again, though not at a uniform rate, and died only when they were markedly smaller than the spores. The smallest healthy cells seen measured no more than $4 \times 3\,\mu$. The residual unaggregated cells in ordinary plates behaved in the same way, but there were far fewer of them. Very occasionally cells have been seen with a diameter greater than $28\,\mu$—that is, as large as the so-called I-cells in *D. discoideum* (Sussman & Ennis, 1959).

THE FOUNDING OF A CENTRE

In low-density cultures on ordinary plates a centre could often be detected when it comprised what appeared to be a single round cell with a few elongated cells attached to it as spokes. However, in other cases, when first seen it was a clump of two or three cells which, though it might gain occasional recruits, did not attract other cells vigorously for some time. Very often, even after a centre had grown to one or two dozen cells, a differentiated core was recognizable—spherical and projecting above the level of the rest—though it was difficult to be certain whether it was a single cell or not.

Because aggregation did not start spontaneously in darkness, one had only to illuminate a virgin coverslip culture when convenient and scan it for a relatively short period in order to watch it produce its first centre. Both this and subsequent ones could usually be seen to be started by single cells; these have been called *founders* (Pl. 1*d*, *e*). A founder varied somewhat in its appearance at the time it became active. In many cases it was oval or almost completely circular in outline and stationary, and either lacked extensive pseudopods or was surrounded by a hyaline skirt of any width up to a maximum roughly equal to the radius of the central granular mass. Because it was less expanded, it frequently appeared smaller than most or even all of the cells that responded to it, and also darker in negative phase contrast. Often its contractile vacuoles were smaller and more numerous as in dividing cells. In other cases the founder was slightly irregular in outline, though not markedly elongated, possessed a few, small, local, hyaline pseudopods, moved very sluggishly, and could not be distinguished by its vacuoles.

There was further variation in the rate at which it attracted other cells. At one extreme—most common if it was round—it abruptly began to affect its neighbours over a considerable area. These elongated towards it within a few minutes, though to very different extents: one responding cell actually measured $42 \times 4{\cdot}5\,\mu$, whereas in others the ratio of length to breadth might remain little more than 1. The first to reach the founder began to encircle it, either in one direction (Pl. 1*d*) or—becoming temporarily Y-shaped—in both. Whether it was able to surround it completely depended partly on their relative sizes, and partly on how soon further cells arrived, for these competed for the founder's surface. Such intimate contact was established that a 2- or 3-celled centre could sometimes be mistaken for a single giant cell. Occasionally, perhaps 5–10 min. after starting to attract, a strong founder became less circular and less refractive and stopped attracting; the responding cells became less elongated and tended to produce pseudopods from other parts of their surfaces; and then perhaps 5 min. later the founder rounded up again, and the others moved towards it. But usually the aggregate continued its rapid growth without interruption (Pl. 1*f*). The founder often remained clearly visible and different until there were a score of cells in the centre. At the other extreme of developmental behaviour a single cell was initially joined by only one or two others, which were rather doubtfully attracted to it and then spread round it quite slowly. The group might not start to attract the outlying cells vigorously for a considerable time, if at all. Until this happened it grew very sluggishly, so that after, say, an hour, even when many cells were apparently available, it might contain but half a dozen, and often no one of them was strikingly different in appearance.

One possibility was that all centres were started by single founders, and that these variant patterns were due to differences in the rate and in the ultimate degree of their differentiation, and perhaps to a lesser extent in the rate at which the other cells became responsive. On the other hand it was possible that in many cases no one cell took a special role, but a clump of cells acted in consort. To try to distinguish between these alternatives, as well as to determine to what extent and for how long, cells that were strongly differentiated as founders while they were still single remained different from other cells after aggregation, it was necessary to devise a means of dispersing a young aggregate to a controlled extent without losing or damaging any of its cells, preferably while keeping them under continuous observation. The principle of the method adopted was to rub the aggregation very gently between the agar and the coverslip by briskly displacing the culture a few microns and relying on the inertia of the agar to yield the requisite relative movement of the two surfaces. The displacement was produced by the spring-operated slide clip of the Zeiss microscope, which pivoted in the horizontal plane; this was simply drawn back a suitable distance and then released. The amount of water between the agar and the glass critically influenced the effect on the cells; it also affected the quality of the microscopic image, an increase producing a deterioration (Pl. 2).

If there was too little water the agar moved with the glass, and the cells, whatever their relationships, were undisturbed. With slightly more water a gentle rap caused all separate cells to withdraw their pseudopods and become either circular or ellipsoidal with the long axis at right angles to the direction of shear. The cells of streams or centres that were not too large or too old behaved in the same way. A small streamless centre became a flattened disk (Pl. 1*g*) with the cells in a single layer, and all, or most of them, apparently completely withdrawn from contact with one another, except, often enough, for a more persistent core of about two to four cells that separated only after repeated or more vigorous rapping. Even if a founder had been distinguishable by shape or contrast in the undisturbed centre it was so no longer, unless by position, immediately after the culture had been struck, for the cells were about equally rounded up. However, after a few seconds they put out pseudopods again. Frequently a founder, having expanded less than the others, could then be recognized by its darkness (Pls. 1*b*, *c*; 2*h*), and also, in so far as there had been room, by the elongation of the surrounding cells towards it (Pl. 1*b*). This treatment in fact revealed founders in many centres where they had not been distinguishable before. It also showed that other cells adhered to them more strongly than to one another, and enabled one to compare the sizes of the cells when they were all rounded up. Certainly the founder might be the smallest in the centre: in one case, for example, it was about one-third the diameter of the largest of the two dozen cells present. But in general it could not be identified by size. Only one founder has been seen to be binucleate.

The cells in a young and strongly attractive centre could be made to round up a score of times in succession, and after each blow they immediately restored the radial organization. But the older a centre, if previously undisturbed, the more difficult it was to reduce it to a monolayer of rounded cells. Rapping a culture more vigorously ruptured some of the cells, but these losses could be easily detected by the cytoplasmic debris and the liberated nuclei. On occasion nearly every cell in the centre was shattered, while the streams were left virtually intact. At first it was thought that this effect revealed a differential fragility, but as it occurred only with the larger centres found in older cultures (in which more cells were available for aggregation not only because of the continuous expansion of the circular feeding front, but because the traces of nutrient in the agar supported very slow bacterial growth) it was eventually concluded that the greater height of the centre was alone responsible, this exposing it to a greater shearing force.

In the conditions just considered, with only a small amount of water between agar and glass, though the cells could round up they were gripped too firmly to change position. With excess water present the shearing force declined, and it was difficult to break up an aggregation completely. But with an intermediate amount the cells not only came apart but changed their relative positions. If these conditions prevailed over the whole culture, the agar disk slid a considerable distance over the glass, the cells dispersed uncontrollably over far too wide an area, and bacteria from beyond the feeding front were scattered everywhere. However, when the total amount of water in the sandwich layer was small enough, it was possible to concentrate most of it in one part of the culture so as to produce the optimum conditions there, while preventing appreciable movement of agar on glass elsewhere. This was achieved by lifting the coverslip off the spacer of the culture chamber, turning it over, depositing a water droplet on the free surface of the agar on top of the desired area, and then re-inverting the coverslip and resealing it in place. The weight of the hanging drop increased the gap between agar and glass in the area directly above it, so that when the culture was rapped aggregated cells in the middle of this area moved apart but remained within the microscope field, cells near the edge of the area rounded up but did not move, and cells further away were unaffected. As the droplet was absorbed the dispersed cells were pinned down, and after a time more water had to be added if they were to be dispersed again (Pl. 2). But if the agar was saturated with water this interval was increased, and the cells could be repeatedly dispersed without further adjustment (Pl. 3).

In those cases in which cells responded strongly to a founder while it was solitary, the first one to arrive could immediately be detached again. Any cells that remained no more than a few cell lengths away from the founder normally then re-oriented towards it, often within seconds. If a rapidly growing centre was first disturbed when it contained a dozen cells or so, it shed most of the peripheral ones very easily; but usually a small core, comprising the founder and the cells directly in contact with it, had to be rapped repeatedly before it dispersed. If the culture produced no more than about a dozen centres during its initial outburst there was sufficient time to analyse each of them in turn when it became strongly attractive; even with those in which this happened after the one-cell stage, re-orientation following mechanical dispersal—except when it did not occur at all—was regularly towards a single cell.

Knocking the ordinary cells off the substratum and separating them greatly stimulated their production of pseudopodia. Often these were thrust out with such vigour that the cells moved in the opposite direction. Both filopodia and lobopodia emerged in any plane from any part of a cell's surface and thus gave it a highly irregular shape (Pl. 3). The cells straightened themselves out, to a large extent, when exposed to an adequate acrasin gradient, but even then often responded with more than one large pseudopod (Pls. 2*a*, *b*, *c*, *e*; 3*b*, *d*, *k*). (U- or Y-shaped aggregating cells were found in undisturbed cultures, though much less commonly). In contrast to all or nearly all the other cells a founder remained small in diameter and formed very few and very sluggish pseudopods, even while unattached; and as it settled down again it expanded slowly and slightly without changing position. The movements of disaggregated cells require finer analysis by time-lapse cinephotography, especially during the period before their re-organization by the founder. The first cells to resume obvious chemotaxis were those nearest it (Pl. 3*a*, *d*, *e*, *m*, *n*). It was most impressive to see them shoot cylindrical jets of cytoplasm directly towards it and, until they managed to anchor themselves, continually correct their aim as they lurched about. If there was sufficient water, gentle rapping would detach the cells without making them round up. With care, an elongated cell close to a founder and pointing directly at it could be made to spin round through exactly 180°. Sometimes it re-oriented by a U-turn; sometimes it first rounded up before sending out a new pseudopod towards the founder; but not infrequently it produced a new dominant pseudopod from the side of the original rear end, or evenly simply reversed its direction of movement, without ever having lost its elongation. Those disaggregated cells further away from the founder responded later than the nearer ones and to a large extent trailed them. This attraction of distant cells by proximal ones produced streams as in primary aggregation; and the markedly non-radial orientation of many of the cells, clearly seen in Pls. 2*e* and 3*c*, demonstrated that this relayed attractiveness could rival the founder's direct influence only a few cell lengths away from it; though, of course, it did not show that the founder might not be considerably more attractive than any one peripheral cell.

Vigorously rapping a culture when there was little water present often burst some of the cells, and on several occasions these included a young centre's highly active founder. The surviving cells of its group formed clumps of varying permanence, joined another aggregation, or wandered off by themselves. These results were extended by using a method by which any cell could be killed as and when desired. By replacing the ordinary microscope lamp by a mercury-vapour lamp and by stopping down its iris diaphragm, the centre of the field could be irradiated both intensively and exclusively. The chosen cell was alone introduced into this area while protected by light and heat filters; when they were removed it died within a few minutes. Any other cells that began to crawl into the area immediately withdrew their pseudopods and remained unharmed. To eliminate complications due to the relayed influence of other centres it was necessary to work with a sufficiently young and sparse point-inoculated culture and to wait for it to produce its first founder. If this was immediately killed, and the culture at once returned to darkness, the residual cells did not reaggregate. But if it was left in the light a new founder eventually did appear, though not necessarily from within the original group. Non-founder cells could be killed in large numbers without affecting the aggregation of the survivors.

It was noted incidentally that if a young, intact centre was heated it sometimes happened that, even if it had thereby been so severely damaged that all its cells had rounded up permanently and become swollen with enormous vacuoles, nevertheless, fresh cells were still attracted to the founder and reached it by nosing their way between the moribund and immobilized ones.

If the founder was not killed or damaged, in most cases it was able to re-organize the others after repeated dispersals, even if a few new cells were allowed to join the group each time (Pls. 2, 3). As the aggregation grew it became increasingly necessary to 'egregate' the founder at each dispersal, i.e. to remove it from the group of separated cells and leave it at such a distance that though they could be reattracted by it, none of them would make contact with it while merely flattening out again on the substratum. It thus became perfectly clear whether or not they did re-orient towards it. Egregation was achieved simply by rapping the culture about once a second, thus shuffling the cells repeatedly until the founder chanced to occupy the desired position. The radial asymmetry of the new aggregation not only emphasized the continuing dominance of the founder over the whole group but facilitated its renewed egregation; but it was important not to wait too long between successive dispersals, otherwise the cells continued to pack themselves all round the founder as well as bond themselves more firmly together.

In one case a founder re-asserted its authority after each of just over a hundred dispersals, while the group grew to about 200 cells. But sooner or later, after anything between a few minutes and a few hours, the founder lost power, sometimes in competition with other cells, sometimes not, dispersal being followed either by multiple aggregation or by little or none at all. In the former case each subgroup, when it could be broken up without damage, yielded at least one cell resembling the original founder in appearance, and more, less, or equally attractive.

By selecting a low-power field containing a founder and no more than one or two dozen other cells, observing them continuously, and rapping the culture every time a cell began to approach another one, one could eliminate the effects of intercellular contact and readily follow changes in the founder's appearance; though of course it was possible that such continual disturbance seriously affected development, interfering not only with the cells individually but also with intercellular induction and inhibition. Whenever the founder was detached from the glass and floating free or lying on the agar it tended to become and to remain almost completely spherical and very bright in phase contrast. But if it made contact with the glass it adhered to it sufficiently strongly to resist this tendency and to transform itself into a dark disk with or without a central hump. In the course of a few hours, during which the number of separate cells in the field perhaps doubled, some of them, in no particular sequence, whether near or far from the founder, came to look like it. Cells might become circular at almost the same minute as they first came to rest and darkened, or not till some time afterwards. Likewise, they might start attracting their neighbours almost immediately the first visible change occurred or not till an hour or so later; and though with this technique it was difficult to compare their strengths accurately, they might at various times be less, equally, or more powerful than the original founder. At the height of the outburst of activity perhaps 10% of the cells looked like founders (in one count: 229 in 2382); and of these at least two-thirds attracted their neighbours in the same way, most of the remainder being too isolated to reveal whether they were attractive or not. After quite variable periods of up to about 4 hr. after their appearance, all these cells, as well as the original founder, lost their radial symmetry, distinctive tonal contrast, and attractiveness, and began to look and behave like ordinary motile cells, able to respond to other 'founders'; this reverse change too was either abrupt or quite gradual.

EFFECT OF BACTERIA

It is generally agreed that aggregation does not begin until some time after the edible bacteria within reach of the cells have been largely consumed (Raper, 1940). The necessary stimulus is a severe rather than an absolute shortage of food, for aggregating cells may continue to digest the contents of their food vacuoles; they may even ingest a bacterium or two (Mercer & Shaffer, 1959). In old, sandwich cultures in which the cells had been aggregating for some days, one occasionally found quite large numbers of bacteria around the aggregations, and even penetrating between the cells of a stream whenever a gap appeared. And in rare cultures even the first centres might develop in areas that had not been completely cleared of bacteria. In these cases, one might suppose that the bacteria had no effect because some change in the environment or in the bacteria themselves had made them sufficiently inedible. However, if one rapped an ordinary young coverslip culture, in which aggregation had begun in an area completely freed of bacteria, it was possible, if one had previously added the right amount of water, simultaneously to disperse the aggregations and to surround their completely separated cells with bacteria, certainly fully edible to the vegetative cells in the culture, at a density that would have *kept* cells in the vegetative stage. Despite this, the separated cells reaggregated immediately and continued to do so after repeated dispersals.

DIFFERENT STRAINS

The above observations were made at least initially on my own stock of *Polysphondylium violaceum*, which was ultimately derived from Raper's strain V6 some 20 years ago and is now periodically cloned. During a visit to Prof. Raper's laboratory I repeated some of the earlier work both on his current culture of V6, which had been maintained by mass transfer of spores, and on strain V9. The results were substantially similar, and indeed the photomicrographs are of these strains; but the differences were appreciable. In Raper's V6, both in ordinary plates and sandwich cultures, a centre was often started by a single, rounded cell; but if this was egregated repeatedly it usually soon failed to re-organize the cell group, and not infrequently it was unable to re-attract more than a few cells after even the first dispersal. Moreover, quite commonly a centre first became strongly attractive when it was a compact clump of up to half-a-dozen cells, no one of which could be immediately identified as the founder with certainty. Thus, clearly, the average level of differentiation attained by the founders in this stock was lower than in the Cambridge one. In sandwich culture

V9 was indistinguishable from the Cambridge V6, regularly producing founders whose special powers usually persisted during repeated dispersals of aggregations over periods of 1–2 hr. On ordinary plates too, at low densities, behaviour was unaltered. But at rather higher densities the cells initially tended to come in contact, without any definite orientation, in small, loose, single-layered groups, which were evenly distributed over the culture areas involved. This was presumably due to a slight general increase in adhesiveness before centres developed. When they eventually did so, some of the groups acquired a radial organization and attracted outlying amoebae, and single, round, higher cells could often be seen at their centres; other groups dispersed spontaneously or entered the radial aggregations; but even in these cultures, solitary cells outside the early groups differentiated into founders.

DISCUSSION

The most extensive work on the start of aggregation has been that of Sussman and his associates. Working mainly with *Dictyostelium discoideum* they believed that they had established that, at *all* stages of the growth cycle, the population was divided in a fairly constant ratio into two phenotypic classes, *initiators* and *responders*; that responders were unable to aggregate in the absence of initiators, which apparently could arise only during an earlier stage; that an aggregation centre was started by a single initiator cell; and that the initiator cells could be identified with visibly recognizable cells, called I-cells.

We may first compare founders with the published description of I-cells (Ennis & Sussman, 1958; Sussman & Ennis, 1959; Sussman, Sussman & Ennis, 1960). (For completeness, I have included a small amount of information about founders derived from experiments that will be published elsewhere.) When lying undisturbed on a surface I-cells are highly expanded, founders little; I-cells are very flat, founders tend to be highly domed; I-cells appear very much larger than all the other cells, founders often appear smaller than most of them. I-cells produce pseudopods more extensively, more frequently and more vigorously than the other cells and are much more highly motile. Founders produce pseudopods less extensively, less frequently and less vigorously than most of the other cells and are almost or completely stationary. Engulfed amoebae are very common in I-cells; but none have been seen in founders. I-cells are frequently multinucleate; founders have never been seen to be. I-cells frequently divide into three or four daughters, founders always into two. I-cells lose their special properties only after one or more divisions; founders do so without division. Some of the clones derived from I-cells show stable differences from the original population; none of the clones derived from founders have shown differences. I-cells are seen during all phases of the growth cycle and the pre-aggregation stage; founders only immediately before aggregation. I-cells form a nearly constant proportion of the population largely independent of cell density and environment; founders an extremely variable one, highly dependent on density and environment. A considerable fraction of I-cells are left outside the centres they are supposed to have started, so it appears that they are not themselves particularly attractive, but rather induce other cells to become so. Founders are invariably the nuclei of the centres they start and may repeatedly restart centres after mechanical dispersal. I-cells may complete their role, which is presumed to be the secretion of a relatively stable initiating substance, as much as 12 hr. before aggregation; and they may then be removed without effect. Founders act only from the start of aggregation, presumably secreting only unstable factors; and if the cells are separated again and the founders removed even after aggregation has begun the separated cells may be unable to re-aggregate.

Despite these differences it might be supposed that founders and the I-cells are simply two kinds of initiator cell used by different species. However, it is now clear that the I-cell is not an initiator cell even in Sussman's haploid stock of *D. discoideum* strain NC4 on which the original I-cell observations were made, and it is also doubtful to what extent it is a separate class of cell: other workers have found that the presence of cells in the I-cell size-range is not in the least necessary for the aggregation of small isolated populations, that they do not form any fixed proportion of the population, that their size range is not discontinuous with that of the other cells, and that they may not show any of the other diagnostic characters that they are all supposed to possess (Konijn & Raper, 1961; Gerisch, 1961; Shaffer, 1961). Moreover, there is considerable internal evidence in the original papers that the I-cells do not have the role ascribed to them (see Shaffer, 1961*a*).

As there has been considerable discussion in recent papers about the extent to which a cell's size may be taken as an indication of its ploidy, it may be noted incidentally that in a haploid strain the difference in size between starved dwarf cells and ordinary feeding or pre-aggregation ones is far greater than that between these and I-cells.

It might perhaps be thought that Ennis & Sussman made a simple error of identification about the I-cell, and that in reality it is the founder that is the initiator cell in the sense used by Sussman. This is not the case. Founders are not present in a definite ratio to the other cells largely independent of the environment both cellular and non-cellular. They do differentiate at the stage they are needed, namely, immediately before aggregation begins; and there is no evidence that they are latently different before this, or that there is any precise limit to the number that the population can produce—certainly there may be several times more of them than the number of initiators implied by Sussman's (1958) preliminary experiments on *Polysphondylium violaceum*. Moreover, the initiator-cell hypothesis is no longer tenable even in *Dictyostelium discoideum*, though we can only briefly indicate here why this is so.

Sussman & Noel (1952) found that the ratio of centres to cells reached a rather sharp maximum at a particular cell density, and held that this equalled the proportion of initiators in the population. But from their data we can calculate that the number of centres *per unit area* remained *constant* over a wide range of cell densities.

Actually, Arndt (1937) long ago noted the constant density of aggregations and fruiting bodies in *D. mucoroides*, and Bonner & Dodd (1961) have recently made counts confirming this in five species. What it implies is that established centres inhibit others from developing within a certain average distance of themselves. In fact, I shall present evidence elsewhere that in *Polysphondylium violaceum*, aggregations secrete a diffusible factor that prevents cells from differentiating as founders, even without switching them into being stream cells. Inhibition by itself will adequately explain the maximum centre/cell ratio. Thus there is no need to suppose that this corresponds to a fixed proportion of initiators.

Sussman & Noel (1952) held that the failure of a certain proportion of small populations to aggregate was due to the absence of initiator cells and that this thus proved the pre-existence of cellular heterogeneity. However, it did not do so; these observations only measured the probability of aggregation in given conditions. Moreover, other workers have found the probability to be vastly greater (Konijn & Raper, 1961; Gerisch, 1961)—at least ten to forty times more initiators than were supposed to exist would have been needed to account for their results. In any case the original observations cannot be explained by Sussman's hypotheses (see Shaffer, 1961).

The remaining point about the initiator was the proof that it was a single cell. This was based on counts of the numbers of centres formed by mixtures of wild strain and mutants (Sussman, 1952). However, the argument was not valid (see Shaffer, 1961). This review and the papers of Konijn and Raper, Gerisch, and Bonner and Dodd, should be consulted for more detailed critiques of the initiator and I-cell question.

Thus the observations on the *P. violaceum* founder provided the first direct and unequivocal proof that aggregation centres could be started by single cells. But we still cannot be sure even in this species, and even at the low densities at which direct observations can be made, whether *all* centres are. Founders vary in the rate at which they differentiate; in their ultimate degree of differentiation as expressed both in their appearance and their powers of secretion; and, in the experimental conditions used, in the length of time they retain their differentiation, and the rate at which they dedifferentiate. Obviously, the less developed they are the more difficult it is to detect them. If none of the cells in a very small centre is morphologically differentiated, and if after being separated none of them is attractive, we at present have no means of deciding whether one of the group was a poorly and very transiently differentiated founder or whether a number of interacting cells took an equivalent role. What is needed is some test for founders that can be performed without disturbing the cells. Time-lapse cinephotography may be of some help.

What of other species? In the three large *Dictyostelium* species the cells form small clumps before they show sustained radial orientation (Ennis & Sussman, 1958; Shaffer, 1959; Bonner, 1960). A small number of fractionations of *discoideum* clumps and young centres did not reveal any highly differentiated founders, the dispersed cells being strongly re-attracted only after a long delay during which they reformed small clumps (Shaffer, 1959). But it was not clear (i) if there *were* single-celled founders, which differentiated rather slowly, perhaps were active only at intervals, and especially after being disturbed did not secrete again for a considerable time; and clumps formed first because the cells happened to have become slightly adhesive; or (ii) if in some or even all cases, collaboration between a group of adherent cells was essential. By carefully plotting individual cell paths in *D. purpureum*, Samuel (1961) subsequently found that in the earliest stages of an aggregation the separate cells oriented periodically towards one particular *motile* cell, which could not otherwise be distinguished by its morphology or behaviour; and he compared it to a *violaceum* founder. Because of the labour involved, he was not able to analyse enough aggregations to determine how regularly this happened. But clearly we may expect to find, in other species, types of founder extending the range of variation seen in *P. violaceum*.

The founder triggers the chemotactic relays; it not only attracts its neighbours it induces them to become more adhesive and to attract their neighbours and induce *them* to attract theirs (Shaffer, 1956, 1958). There is as yet no evidence that the founder's secretion is qualitatively different from that of the other cells in an aggregation; and although these initially adhere to it more strongly this may well be only a temporal difference. So one might assume that all the cells followed essentially the same developmental pathway; that the founder was merely the first to do so, and hence was able to concentrate all its energies on secretion; and that the others were prevented from doing this, until they reached the centre, simply by being kept on the move. However, if the founder were induced to secrete attractor by the same extracellular factor as made the other cells do so, this inducer would have to be made by the whole population spontaneously, the founder being the cell with the lowest threshold. But cells cannot regularly be made to aggregate simply by heaping them together (Shaffer, 1958); and yet in low-density cultures a founder commonly becomes active at a considerable distance from its nearest neighbours. Further evidence that the developmental pathways are quite different is that both darkness and sandwiching the cells somewhat inhibit the differentiation of founders, and in combination they do so very severely; but neither factor hinders the growth of streams, once started, and in fact sandwiching slightly facilitates the operation of the relays. The special nature of a founder's activity is made particularly apparent by a culture plate in which the cells are everywhere so numerous as to form a continuous layer and yet, despite the enormous possibilities of mutual stimulation by secretion and contact, are unable to start building true aggregates; yet if illuminated they would readily do so when at a density a hundred or more times lower.

The situation in *Actyostelium* is still more striking (Shaffer, 1959, unpublished obvervations) for here sandwiched cells cannot start to aggregate spontaneously even when illuminated, and yet when triggered by a homologous centre at the edge or on top of the agar overlay they will form streams which are commonly far more extensive than those they make when not in a sandwich. I have pointed out that such sandwich cultures may be used as a test system for the relevant component of acrasin. For although it is possible that streaming might be induced by many unspecific metabolic stimuli, this is unlikely, because the facilitation of relay activity shows that the agar overlay does not induce *general* metabolic depression. This test has already been exploited by Hostak (1960), who has found streaming inducible by various alkaloids and by some but not others of the steroids that Wright & Anderson (1958), using my first acrasin test, discovered were active on *D. discoideum*. I have not found any members of the Dictyosteliidae to be completely prevented from founding centres in illuminated sandwich cultures, but several species are appreciably inhibited.

If the founder's development is different, it is easier also to explain the fact that after mechanical dispersal in the early stages of aggregation it can resume secretion immediately, whereas the other cells, despite their having previously secreted an important amount of attractor, may still need it to re-induce them to secrete, and in its absence may fail to re-aggregate. Thus the peripheral cells have to be stimulated for some time by the external inducer before they become strongly adhesive, and their powers of secretion more resistant to disturbance.

A further question is whether all the cells in a genetically homogeneous population would in time differentiate like founders if not inhibited by unfavourable conditions or previously established aggregations. We may tentatively suppose that at some stage in this differentiation a particular biochemical configuration has to appear spontaneously within a cell, and that the probability of this happening will depend on its physiological age and on both its cellular and non-cellular environment.

The fate of a founder in normal development is unknown. In all the experimental conditions used so far in which it can be kept under observation, it invariably sooner or later returns to looking and behaving like an ordinary unaggregated cell, and it can then orient in an acrasin gradient. The siting of new centres in sandwich cultures re-illuminated after their original aggregates have disintegrated suggests that any cells that have secreted acrasin for a considerable period, former founders included, are much less likely to become founders than are those that have not done so.

Observations have been made that are relevant to three other aspects of slime-mould behaviour. Bonner (1950) found that if the acrasin gradient was reversed, a *D. discoideum* cell responding to it would invariably re-orient by making a U-turn, if it remained elongated. However, although there is certainly a strong tendency for the polarity of aggregating cells to be preserved, they do sometimes directly reverse their direction of flow without having first rounded up or shortened.

The question of whether cells that have once entered the social phase can be made to return to a solitary, vegetative existence by resupplying them with food has been disputed since 1880 (see Raper, 1940). Arndt (1937) found that most of the cells taken from an aggregation or developing fruiting body of *D. mucoroides* would immediately re-aggregate, even if they were completely separated and the original organization was totally destroyed, provided that they were not too scattered and the added bacteria not too numerous, although a few cells always did start to feed. He concluded that once food shortage had sufficiently altered a cell's properties, food became less effective in influencing behaviour, though it could still be decisive if present in sufficient quantity. But Raper (1940) found that if aggregations and migrating aggregates of *D. discoideum* were covered with bacteria, any cells that were separated, or simply stirred up so that they lost their original positions, returned to the vegetative stage. Observations reported in the present paper agree with Arndt's and support his interpretation. However, it is still undetermined whether aggregated cells that are *not* mechanically dispersed will remain unaffected by a concentration of ambient bacteria higher than can be accounted for simply by their physical inaccessibility.

Finally, we may consider the still-vexed question of sexuality. Wilson & Ross (1957) conceded it was possible that not all haploid cells had to pass through a sexual phase, but nevertheless believed that the majority of them did so, forming zygotes by engulfment just before entering aggregations. These authors examined two isolates of *Polysphondylium violaceum*, including one of Raper's used in the present investigation, as well as three other species, and in *all* of them found meiotic divisions 'soon after the first zygotes formed a centre of aggregation'. However, in my cultures, despite the excellent conditions for observation, and the possibility of dispersing an aggregate and examining all its component cells, it has been extremely rare to see any cell dividing, or a healthy cell engulfed by another one, either within a centre or a stream or during their formation. Ross (1960) has recently reported that in *Dictyostelium discoideum*, sexual activity occurs both less commonly and less regularly than he previously found. Although neither Wilson nor Ross have ever suggested anything of the sort, one explanation of the founder's different development might be that it was the sole zygote in an aggregation. We cannot completely exclude this possibility without taking time-lapse films, but the founder's size and its conversion into a normal cell in the absence of cell division make it improbable; it is also extremely unlikely that syngamy could have escaped detection.

SUMMARY

1. In cloned populations aggregation centres are started by the differentiation of single cells called *founders*. These attract their neighbours and induce them to attract theirs.

2. Founders vary in their degree and rate of differentiation. Those most highly differentiated are rounded up, stationary, and highly adhesive. They are very different from the 'I-cells' and hypothetical 'initiator' cells described in the literature.

3. A technique has been developed for reducing a centre to a monolayer of cells and dispersing them to any desired extent. This reveals the founder if not previously apparent. The original founder will repeatedly re-organize a group of cells subjected to successive dispersals, though additional founders may eventually differentiate among them.

4. If a young aggregate is dispersed, the founder killed, and further founder differentiation inhibited, the cells do not re-aggregate.

5. If kept separate a founder invariably dedifferentiates within a few hours.

6. Illuminating a culture produces an outburst of founder activity. If the cells are sandwiched between agar and glass this outburst is delayed. And if sandwiched cells are kept in darkness the differentiation of founders is very severely inhibited, even at extremely high cell densities. In the same conditions aggregation streams once started grow extensively. The developmental pathway of the founder appears to be quite different from that of the other cells in the aggregate.

7. Re-aggregation after dispersal is not inhibited by a density of edible bacteria that would suffice to keep cells in the vegetative state.

8. A separate, elongated cell oriented by an acrasin gradient may directly reverse its direction of movement if the gradient is reversed.

I should like to thank Prof. K. B. Raper for his hospitality and Mr T. Konijn for his assistance while I was working in Wisconsin University. This research was partly supported by grants from the National Institutes of Health, U.S. Public Health Service, and the Nuffield Foundation.

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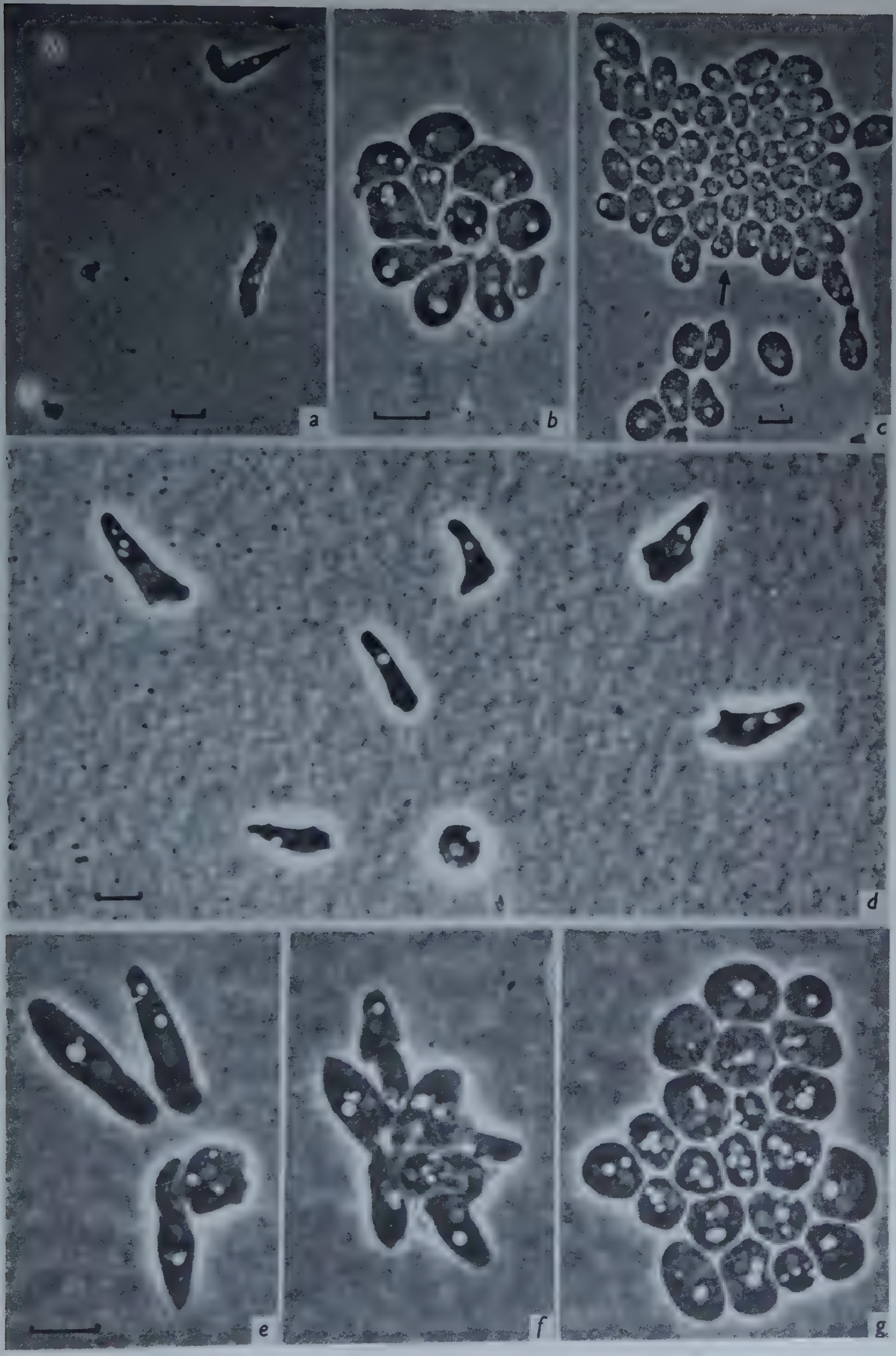
EXPLANATION OF PLATES

PLATE 1

All scales 10 μ. (*a*, *e–g*) strain V6; (*b–d*) V9. (*a*) Two spores, two starved 'dwarf' cells, and two cells of average size that have wandered back into this area after feeding. The back of one cell is drawn out into a very fine 'tail' (arrowed). (*b*) A centre, with little water present, a few seconds after being rapped. The founder is in the middle still rounded up and somewhat darker. Most of the other cells have already put out pseudopods and elongated towards it. (*c*) A larger centre reduced to a monolayer as in (*b*). Pseudopods are re-appearing. The founder, small and dark, is the third cell above the arrow. Note the reduction in cell size from the periphery inwards. (*d*) The start of aggregation. A founder that has just become active. (*e*) Another newly active founder. One cell has already reached it and begun to flow round it. Two more are about to arrive. (*f*) The same aggregation 10 min. later. The round, dark founder is still clearly recognizable. (*g*) The same aggregation when a little bigger, immediately after rapping. All cells still with their pseudopods withdrawn, and the founder not obvious.

PLATE 2

Scale 10 μ. Strain V9. Some of the repeated dispersals of a group of aggregating cells, showing the effect of different amounts of water in the sandwich layer when the culture is rapped. (*a*) Enough water present for the cells to move freely apart. The 'bright' founder (arrowed) has already begun to



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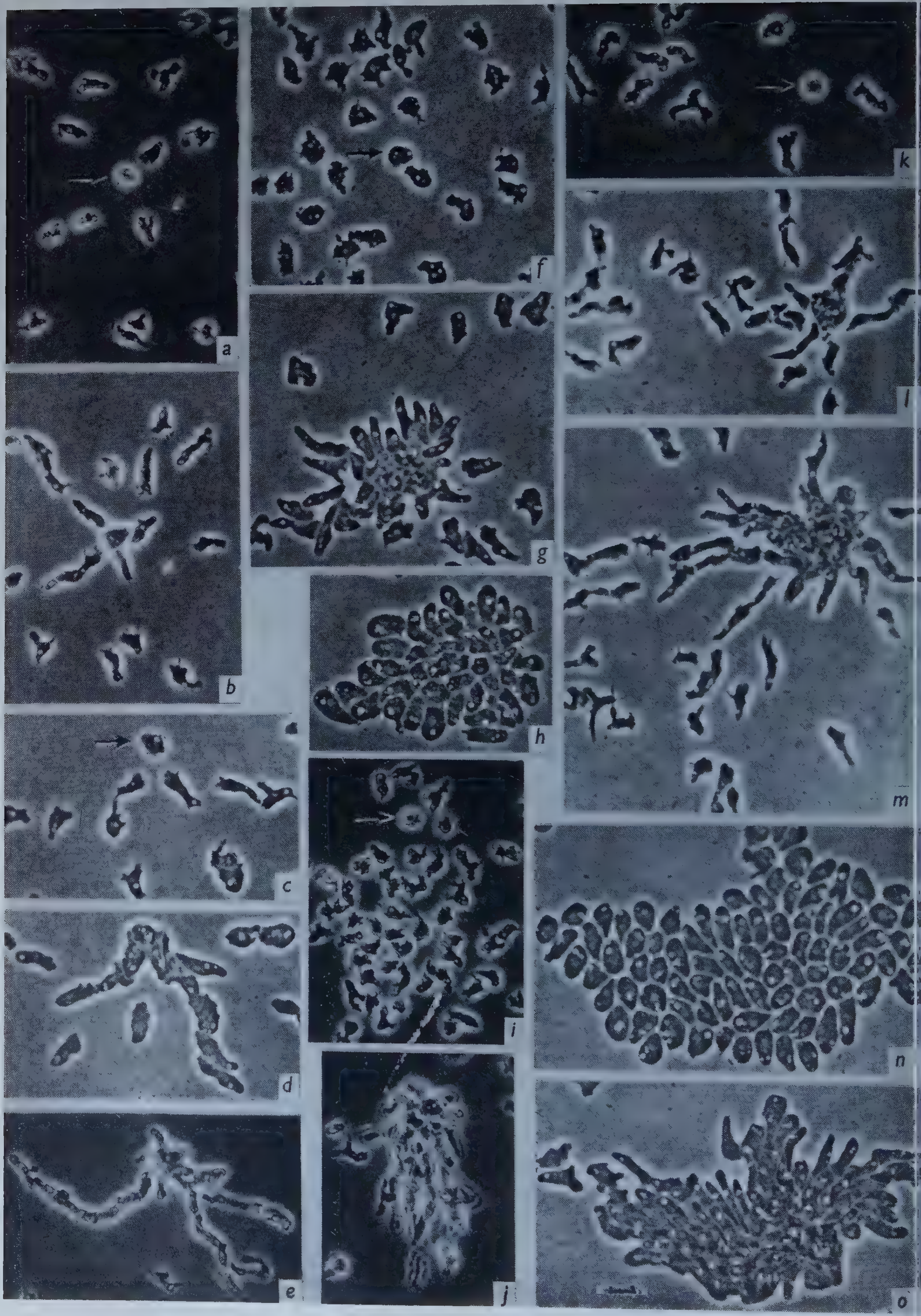

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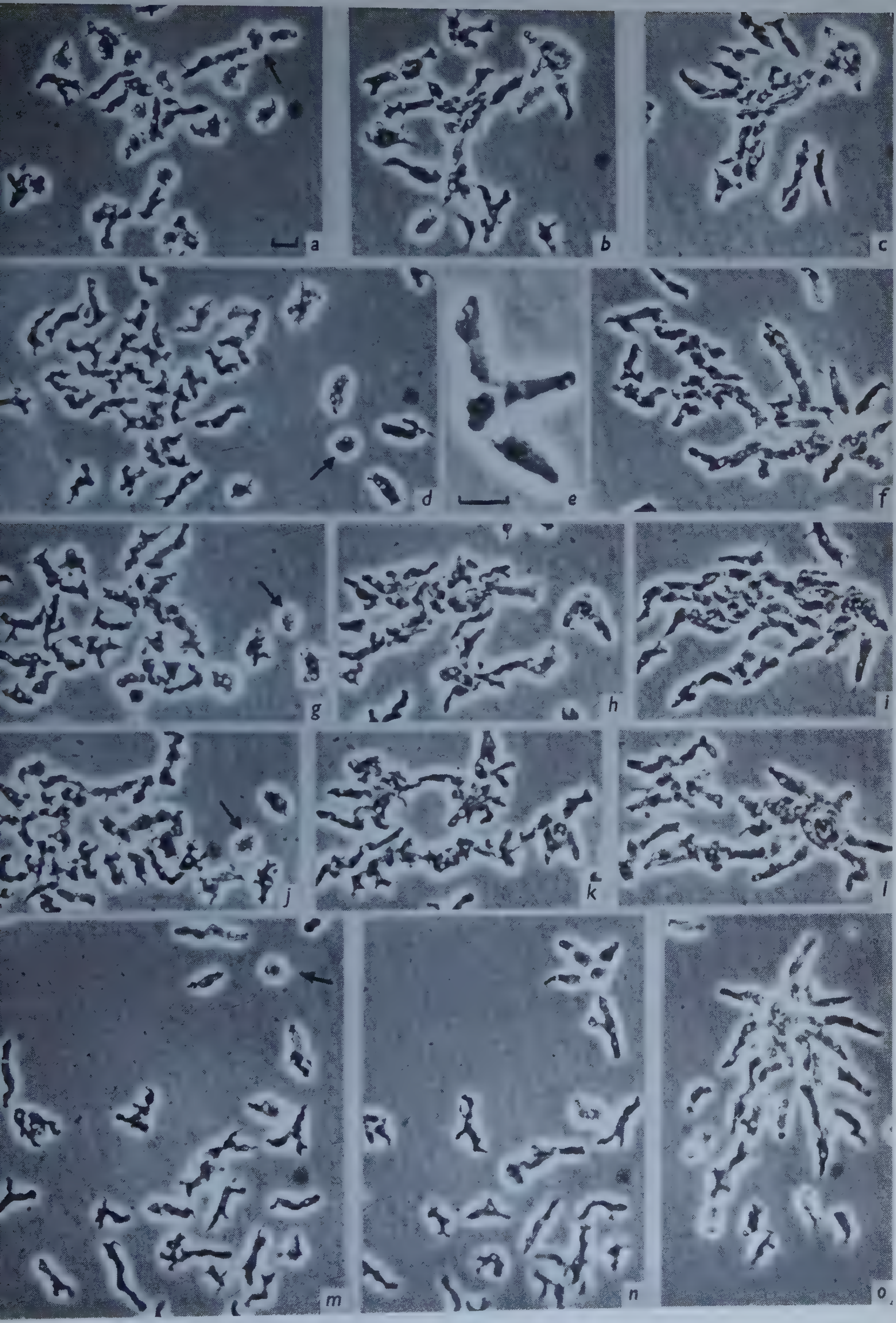

B. M. SHAFFER

re-attract the nearest cells. (*b*) Re-aggregation without further disturbance. (*c*) Renewed dispersal, and egregation of the founder. The water is being gradually absorbed, and the cells flattened. (*d*) Still less water, more flattening, and better optical conditions. The cells follow one another as they re-aggregate. (*e*) Re-aggregation further advanced. Stream at left almost semicircular. More water added to permit subsequent dispersal. (*f*) Cells dispersed and some water re-absorbed. (*g*) Re-aggregation; less water. (*h*) Culture rapped without adding more water. The cells, having rounded up and formed a monolayer without dispersing, have spread out again and elongated within the limits of the space available. The founder is clearly recognizable by its position and darkness. (*i*) Water added, and the aggregate dispersed. Founder bright and nearly spherical; other cells already considerably expanded. (*j*) Re-aggregation in the presence of a lot of water. (*k*) Renewed egregation. (*l*) Re-aggregation. Water being absorbed. (*m*) Further re-aggregation; cells falling into line behind one another even before re-establishing contact. (*n*) Little water. Aggregate reduced to a monolayer by rapping. Recovery not so advanced as in (*h*) and founder less obvious. (*o*) Re-organization of the aggregate.

PLATE 3

Scales 10 μ. Strain V9. Records of five of a much larger number of re-aggregations of a group of cells towards the same founder (arrowed). In each row of photographs, the one on the left was taken soon after egregating the founder, that on the right shows the amount of re-aggregation that was allowed to occur before renewed dispersal.

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RECEPTOR POTENTIALS RECORDED FROM SENSILLA BASICONICA ON THE ANTENNA OF THE SILKWORM LARVAE, *BOMBYX MORI*

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Several authors have reported on electrical activities of antennae of insects in response to various olfactory stimuli (Boistel, 1953; Roys, 1954; Boistel, Lecomte & Coraboeuf, 1956; Schneider & Hecker, 1956; Schneider, 1957). Dethier (1941) studied the sensilla of the antenna of lepidoptera larvae, and assumed that the 'large' sensilla basiconica were olfactory organs.

One of the authors (Morita, 1959) recorded slow potentials directly related to occurrence of impulses (i.e. receptor potentials) from the side wall of the contact chemosensory hair on the labellum of the blow-fly, *Calliphora vomitoria*. The 'large' sensilla basiconica on the antennae of lepidoptera larvae are far smaller than the labellar chemosensory hair, but not so small as to prevent application of the method available for the labellar hair. In the present study, using this method, electrical activities of the sensillum basiconicum of the silkworm larva were qualitatively analysed.

METHODS

As described by Dethier (1941), the antenna of lepidoptera larvae consists of three segments: the first segment from the base of the antenna contains four sensilla campaniformia; the second has one sensillum campaniformium, two sensilla trichodea and three sensilla basiconica (two large and one small); the third has one sensillum styloconicum and three sensilla basiconica (one large and two small). Dethier (1941) described the large sensillum basiconicum as innervated by more than twenty sensory neurons. This type of sensillum on the antenna of the silkworm larva was used in the present study. It may be assumed that the histological structure of this sensillum of the silkworm larva is the same as reported by Dethier, because this larva belongs to the order Lepidoptera.

The arrangement for recording electrical activities of chemosensory neurons was almost the same as used by Morita (1959). The head of the fifth-instar larva of the silkworm, *Bombyx mori*, was isolated at its base. As shown in Fig. 1, a platinum wire, which was thrust into the base of the antenna from the cut end, was grounded through a balancing voltage (*BV*) and calibration pulse sources (*CP*). A glass capillary (*Cr*) filled with 0·65 M-NaCl with a tip diameter of about 1 μ was used as a recording active electrode, the tip of which was inserted into a 'large' sensillum basiconicum on the antenna by means of a micromanipulator. Electrical activities of the sensillum were fed into an amplifier through a platinum wire which was dipped into the 0·65 M-NaCl solution in the capillary (*Cr*).

By means of a hydrostatic pressure, air which flowed through an activated carbon filter and a flexible vinyl tube was directed from an opening of a glass tube (*Ca*, about 50 μ in diameter) toward the tip of the sensillum. Although the vinyl tube was washed with running water for a long time after cleaned with detergent, it might not have been completely odourless. For this reason the response to a given chemical stimulus was always confirmed by a control experiment as described below. The pressure, which drove the air out, was produced by water at different levels in two communicating bottles. The rate of air flow could be regulated with a pinch cock which was fitted to the vinyl tube. The tip of the 'air-tube' (*Ca*) was placed at about 0·3 mm. from the sensillum. A stimulating capillary (*Cs*), 10–30 μ in tip diameter, was placed near the tip of the 'air-tube' (*Ca*) perpendicularly to the air current as it flowed on to the sensillum. The olfactory stimulation was applied by exposing the tip of capillary (*Cs*) filled with an odorous substance to the air current with the aid of an electromagnet, while an empty capillary was used in control experiments. The beginning and the end of an electric current through the electromagnet are indicated by arrows in each record. The chemical stimuli therefore became operative after some delay relative to these marks, which depended upon the rate of air flow.

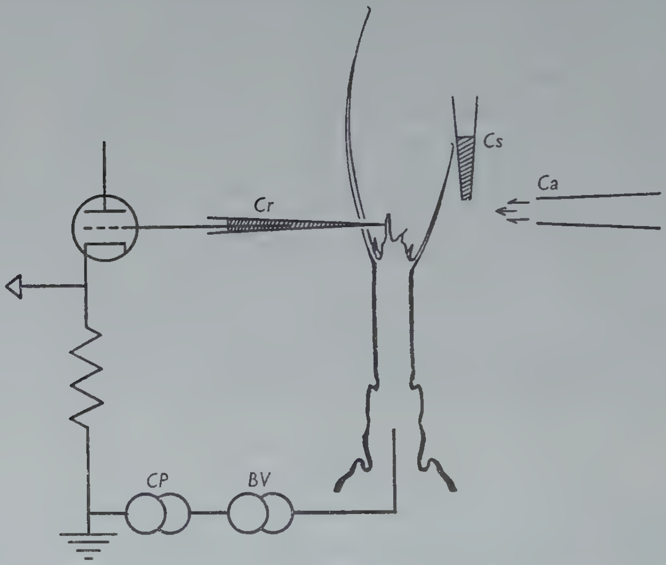

Fig. 1. Experimental arrangements. *Cs*, stimulating capillary containing a stimulus substance, mounted on the moving iron of an electromagnet; *Ca*, capillary used to blow air to the sensillum; *Cr*, recording capillary; *CP*, calibration pulse source; *BV*, balancing voltage source.

Odorous substances applied were *n*-hexanol, $\beta\gamma$-hexenol, *n*-butylaldehyde, acetic acid, butyric acid, etc. The essential oil of mulberry leaves contains all of these substances except *n*-hexanol (Watanabe, 1958). It is known that $\beta\gamma$-hexenol is strongly attractive to the silkworm larvae (Watanabe, 1958). All stimulating substances were used without being diluted with any solvent. The strength of the stimulus was therefore not strictly controlled, but this fact was not serious for the present qualitative study of the chemoreceptors.

The head of the d.c. amplifier used was composed of a 12AU7 cathode follower, its grid current being less than 10^{-12} A. The electrical responses of the sense cells, appearing on repeated sweeps of an oscilloscope, were photographed on a running film. In each record a calibration pulse of +1 mV. (i.e. positive with reference to earth) was added just before the chemical stimulation. In most cases the rectangular wave of 60 cyc./sec. was also recorded simultaneously, and served for the control base-line as well as the time base.

All experiments were performed at room temperature, 26–29° C., and at relative humidity 80–85 %.

RESULTS

Spike potentials

When the tip of the recording electrode was inserted into the sensillum, a sudden drop of potential of a few hundred millivolts appeared and spike potentials were observed. In Fig. 2 some of these spikes are shown. These spontaneous spikes were not different in shape from those occurring upon chemical stimulation. It should be noticed that they are diphasic, positive to negative. This polarity is the same as observed in contact chemosensory hairs on *Calliphora labella* (Morita & Yamashita, 1959*b*; Morita, 1959).

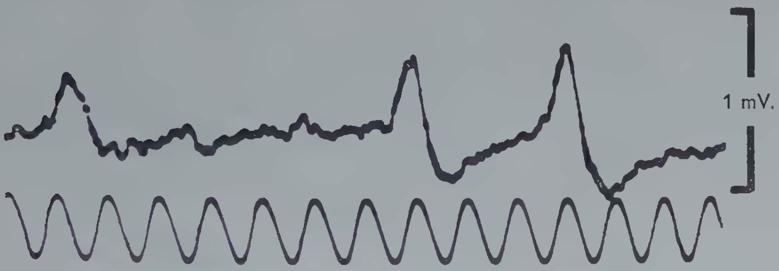

Fig. 2. Spikes recorded with an electrode thrust into a sensillum basiconicum. Time base, 1000 c/s.

Slow monophasic potential

When the tip of a sensillum was exposed to a current of pure air for a brief period an increase in impulse frequency accompanied by a slow negative potential was recorded. In Fig. 3A the beginning of this response is shown on the first sweep, and after cessation of the air current the slow potential is seen to return to the initial level (at the beginning of the fourth sweep). As the sensillum was again exposed to a continuous air current, the base-line and the frequency of impulses attained a new steady level. This steady state was not affected at all by exposing the tip of a dry capillary to the air current (Fig. 3B). On the other hand, a slow negative potential and an increase in impulse frequency were induced if a capillary whose tip was filled with *n*-hexanol was used (Fig. 3C). The impulse frequency and the slow potential returned to their initial level when the capillary was withdrawn from the air current. From these results it may be assumed that the slow potential and the increase in

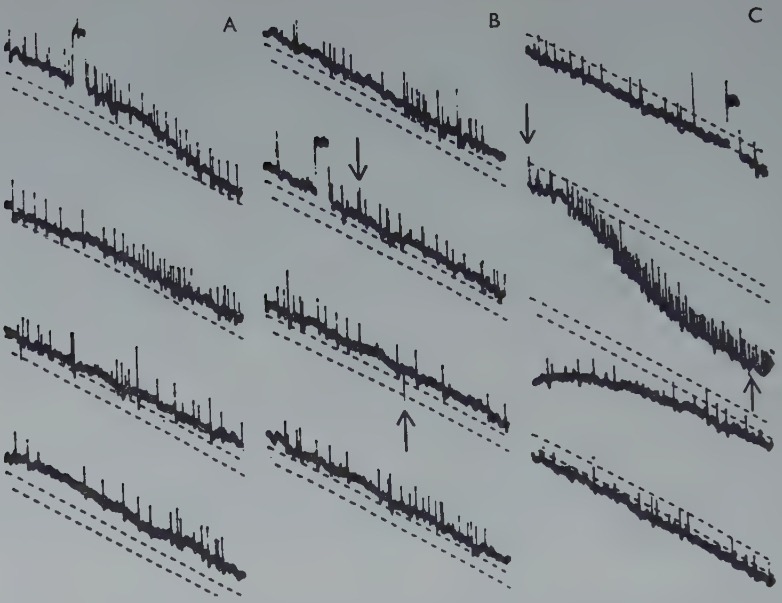

Fig. 3. A, air blown over the sensillum (from the middle of first sweep to the end of third sweep). A clean dry capillary (B, as control) and a stimulating capillary containing *n*-hexanol (C) were exposed to the air current. Arrows show 'on' and 'off' of an electric current through the electromagnet on which the stimulating capillary was mounted. Rectangular pulses of 60 cyc./sec. served also as control base-line. Calibration of rectangular pulse in each record, +1 mV. Unless stated otherwise subsequent figures are presented in the same way.

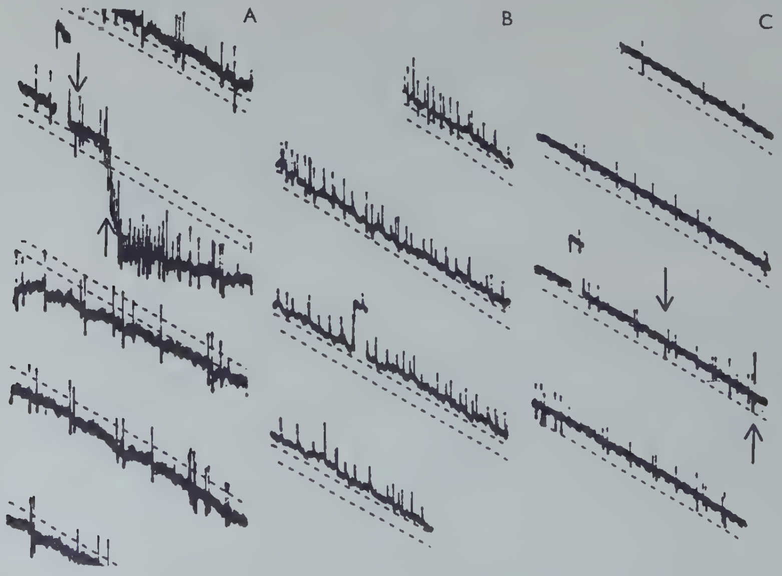

Fig. 4. Application of sulphuric acid to the tip of the sensillum. A, responses to *n*-hexanol before application. B, deformation in shapes of spikes immediately after application. C, responses to *n*-hexanol after application.

impulse frequency were responses to chemical stimulation. The results shown in Fig. 4 also support this assumption. After the response to *n*-hexanol was ascertained to exist (Fig. 4A), the tip of the sensillum was touched with sulphuric acid, and then impulses became monophasic and showed a temporary increase in frequency (Fig. 4B). Thereafter, no response to *n*-hexanol was seen (Fig. 4C) except small spikes which were not related to chemical stimulation, and the origin of which we could not trace. Even then, another sensillum on the same antenna responded normally to *n*-hexanol.

There was no significant difference between electrical responses to *n*-hexanol (Fig. 5B) and to $\beta\gamma$-hexenol (Fig. 5C). Strictly speaking, however, it was almost impossible to compare their effectivenesses because of the difficulty in accurate regulation of the stimulus strength.

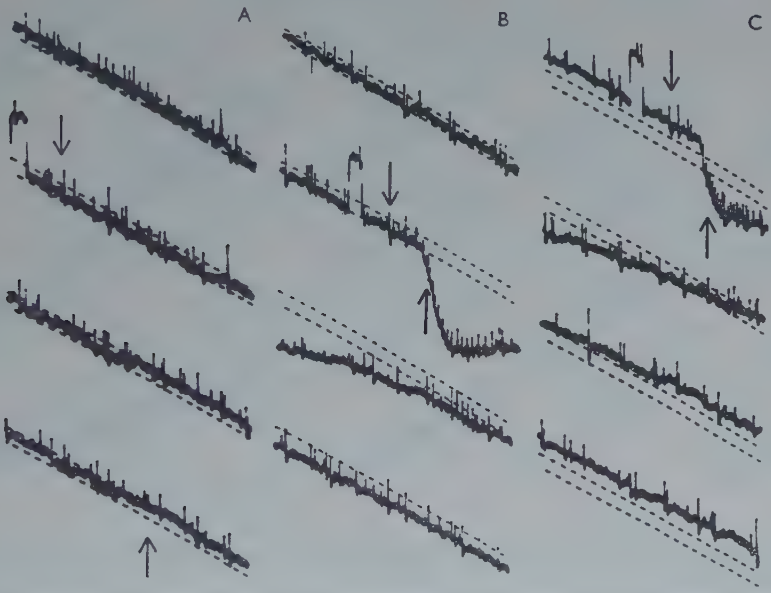

Fig. 5. Responses of a sensillum to $\beta\gamma$-hexenol and to *n*-hexanol. A, dry capillary (control); B, *n*-hexanol; C, $\beta\gamma$-hexenol.

Thus the negative slow potential was accompanied by an increase in impulse frequency in the antennal sensillum of the silkworm larva as observed in the labellar chemosensory hair of the fly (Morita, 1959). At the same time it should be noticed that impulses in the sensillum were discharged without chemical stimulation at a much higher frequency than in the labellar hair.

Slow diphasic potential

In many cases *n*-butylaldehyde induced responses of the same type as described above. In some cases, however, especially with strong stimulation, it provoked a different type of response. Fig. 6 (continuous from *a* to *c*) shows an example of this type which was recorded from the same sensillum as used in Fig. 3. In order to show the individual impulses evoked by the stimulus, a part of the record in Fig. 6 was enlarged and is presented in Fig. 7. The potential declined slowly during the chemical stimulation, but after cessation of the stimulation it ascended gradually to a more positive level than the initial level. While the negativity was maintained, spikes (which were smaller than those evoked by *n*-hexanol) increased in frequency. Except for this period there was no detectable difference in impulse frequency. As can be seen in Fig. 7, small spikes decreased in height with an increase in sustained negativity, and seemed to be masked by noise under deep negativity, gradually growing up again to the original height with the recovery from the negativity. This observation may be explained by the relation established between the strength of the applied electric current and the spike height in contact chemosensory hairs of some insects (Morita & Takeda, 1959; Morita, 1959).

Slow diphasic potentials were recorded on stimulation by some other substances such as butyric acid, acetic acid, etc. An example of responses to acetic acid is shown in Fig. 8: *a*, *b* and *c* in A are the records obtained at the periods *a*, *b* and *c* in B, respectively, which represents the whole course of the slow potential. It was impossible to indicate the time of break of the electric current through the electromagnet because of the disappearance of the artifact. However, it must have been near the middle of the third sweep in A*a*, as judged from reading of the dial in an apparatus for current supply. As shown in this figure, the sustained positivity was much larger in amplitude and much longer in duration than the negativity maintained during stimulation. It was also noticeable that impulse frequency depended upon the rate of change in slow potential rather than upon the magnitude of the potential; that is, impulses increased or decreased in frequency with a fall or a rise of the slow potential, respectively. This tendency was generally observed in the responses to other stimulating substances. An attempt was made to study the nature of this after-positivity later in the same

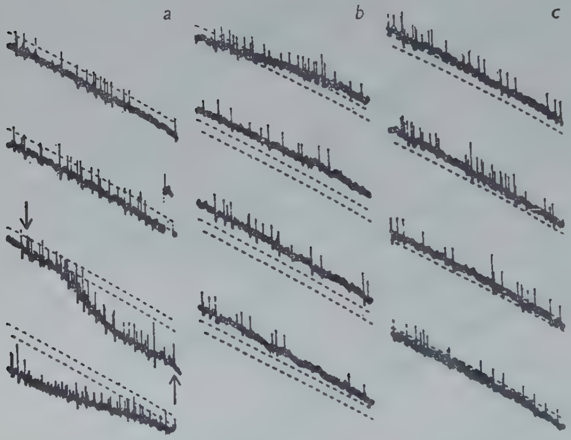

Fig. 6

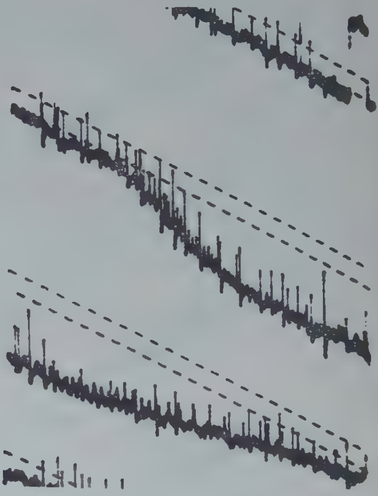

Fig. 7

Fig. 6. Responses to *n*-butylaldehyde of the same preparation used in Fig. 3. Record continues from *a* to *c*.

Fig. 7. Enlarged photograph of a part of the record in Fig. 6, showing the individual impulses evoked by the stimulus.

year, but then all preparations responded without the after-positivity except one responding to acetic acid.

In responses to n-hexanol and $\beta\gamma$-hexenol an increase in impulse frequency was always accompanied by an increase in slow negativity, without exception. This result may be explained by the assumption that the receptor potential evoked near the tip of a sensillum initiates impulses somewhere near its base (cf. Morita, 1959). Diphasic slow potentials cannot be interpreted as simply as above. Moreover, it was observed that impulses increased in frequency during the slow monophasic positive potential. Fig. 9 shows an example of this sort, where n-butylaldehyde was applied. Record A in this figure was obtained with a stimulating capillary of about 10 μ in tip diameter. The slow negative deflexion was maintained while impulses increased in frequency, and no after-positivity was seen. Thereafter, when the stimulating capillary was replaced by another whose tip diameter was 25 μ, the slow positive deflexion appeared, being accompanied by an increase in impulse frequency (Fig. 9 B). Since these results were taken from the same sensillum, it may be assumed that the difference in response between records A and B was due to a difference in stimulus strength. At the same time, these results suggest that n-butylaldehyde might be excitatory for one group of chemosensory neurons and inhibitory for another group in the same sensillum. Further discussion will be given below.

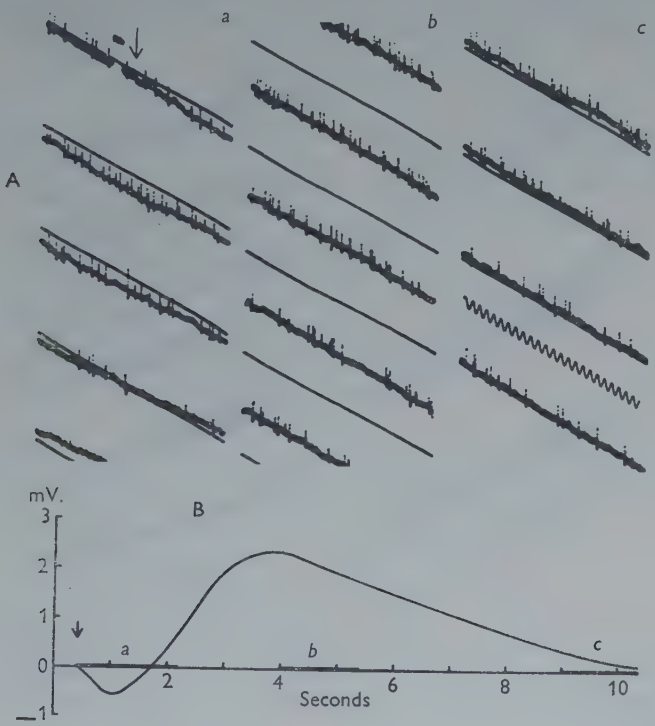

Fig. 8. Responses to acetic acid. Record A shows three parts of the whole response which is plotted in B. Thick lines a, b and c in B correspond to a, b and c in A, respectively. Calibration pulse, +1 mV. Time base, 60 cyc./sec. An electronic switch was used for obtaining dual beams, and one of the beams served as a control base-line.

Effect of anaesthetics

It was observed by Schneider (1957) that impulses recorded from the antennae of the silkworm moth were abolished by ether or chloroform vapour. In the present study impulses were never blocked with a capillary of less than 100 μ in tip diameter containing ether or chloroform, no matter how long the exposure. Ether or chloroform, having a low boiling point, formed a droplet at the capillary tip, and this droplet disappeared because of rapid evaporation; then, another droplet was formed and disappeared in turn and this process was repeated. Fig. 10 (continuous from *a* to *c*) is a part of a record obtained during such an experiment with chloroform. In this figure the arrows indicate the moments when a droplet began to be formed and when the droplet disappeared. The sustained negativity coincided exactly with the formation of the droplet, being accompanied by trains of impulses. A more important fact in this record was alternation in occurrence of trains of impulses; after disappearance of the droplet the sustained negativity returned to the initial level, and as it did so a single train of impulses began to appear. A high frequency of impulses immediately after the disappearance of the droplet and its subsequent decline indicated that this single train of impulses was initiated by the disappearance of the droplet. From this result it may be assumed that chloroform vapour depolarized the receptor membranes of one group of chemoreceptors and initiated impulses in them. On the other hand, the same vapour may have hyperpolarized the receptor membranes of another group; after disappearance of the droplet an after-negativity might initiate impulses in this group of receptors, as observed in labellar chemoreceptors of the fly (Morita & Yamashita, 1959*a*). In fact, concentrated vapour of chloroform induced a positive

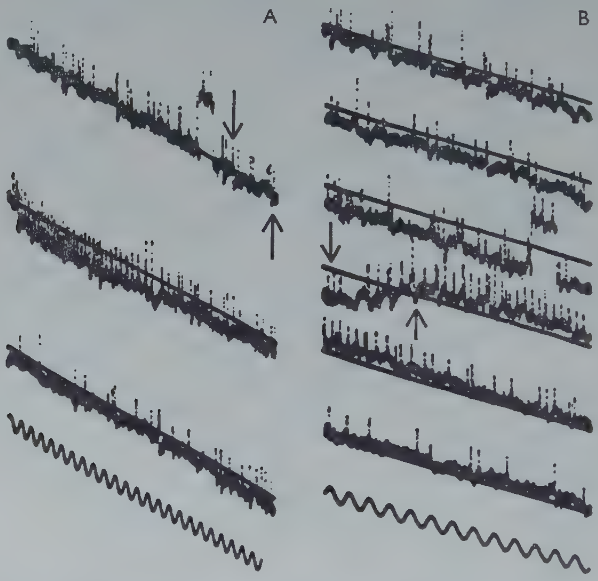

Fig. 9. Responses of the sensillum to *n*-butylaldehyde. In record A the tip diameter of the stimulating capillary was about 10 μ; in record B, about 25 μ. Time base, 60 cyc./sec. The recording equipment as used in Fig. 8.

potential which was maintained during the exposure, while the discharge of impulses was depressed. Fig. 11 (slow potential only was d.c. amplified, and spikes were a.c. amplified) shows a response of a sensillum to a strong stimulation by chloroform vapour. In this case a cotton wick, soaked in chloroform, was brought extremely close

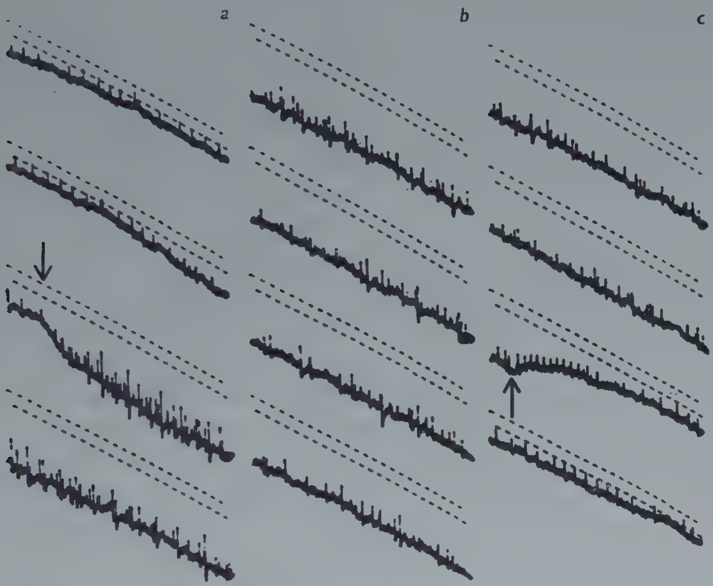

Fig. 10. Response evoked by exposing the capillary containing chloroform to the air current blowing to the sensillum (continuous from *a* to *c*). Arrows, ↓ and ↑, indicate the start of the formation and the disappearance of a chloroform droplet, respectively.

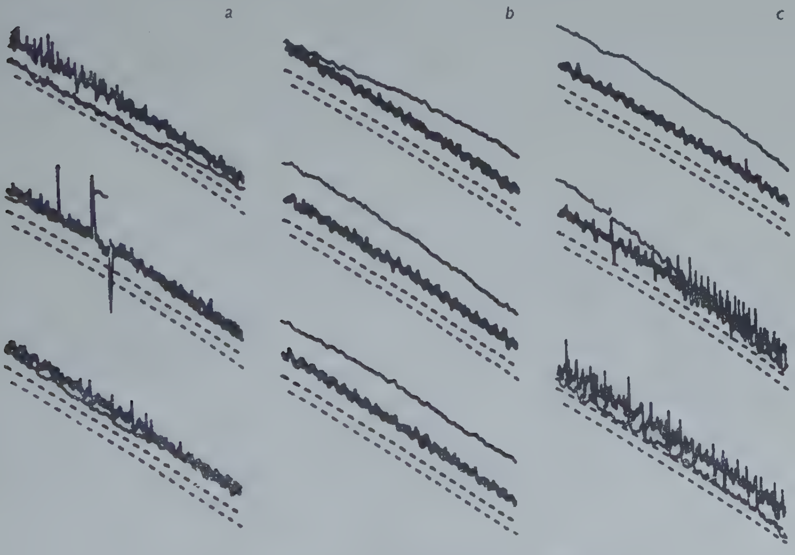

Fig. 11. Potential changes in the sensillum when a cotton wick soaked in chloroform was brought close to its tip for a short time. Continuous from *a* to *c*. Slow potential and impulses were separately recorded. Calibration pulses of +1 mV. are shown on second sweep in *a*.

to the sensillum for a short time. During the sustained positivity there was no train of impulses, suggesting that this positivity represented hyperpolarization of the receptor membrane.

DISCUSSION

Schneider (1957) recorded slow potential changes from antennae of the silkworm moth induced by olfactory stimulation, with a capillary electrode inserted into the antenna; he called this slow potential 'electro-antennogram', and considered it to be made of a number of components. Discussing the relation between the electro-antennogram and the impulse, he pointed out that the peak of impulse frequency did not coincide with that of the electro-antennogram. It was also assumed in the present study that the recorded slow potential was a complex of a few groups of receptor potentials. However, it may be possible to say from the present results that the negative receptor potential augmented the rate of impulse discharge.

As already described, the structural situation seems to be the same both in labellar chemosensory hairs of the fly and in sensilla basiconica of the silkworm larva. In the present paper it has been shown that electrical responses are also similar in the two kinds of sensilla if the same recording arrangement is used. That is to say: (1) Spikes were diphasic, positive to negative. (2) A chemical stimulus generally induced a slow negative potential accompanied by an increase in impulse frequency. (3) A slow positive potential, which could be evoked by application of anaesthetics, abolished all impulses. These results may be explained, as described for the labellar chemoreceptors of the fly (Morita, 1959; Morita & Yamashita, 1959*a*), as follows: chemical stimulants depolarized or hyperpolarized the receptor membranes which were located near the tip of the sensillum. These changes in the membrane potential controlled the rate of discharge of impulses which were initiated somewhere near the base of the sensillum. The initiated impulses were conducted toward the tip of the sensillum as well as to the nervous centre. Accordingly, they were recorded as diphasic (positive to negative) impulses with an active electrode inserted into the sensillum. On application of sulphuric acid to the tip of a sensillum chemosensory impulses became monophasic before the sensillum became completely unresponsive to chemical stimulation (Fig. 4). This is reasonable, because impulse conduction must be blocked at the point near the tip sooner than at the basal region (cf. Morita, 1959).

On the other hand, some of the present records showed characteristics which were not observed in the labellar hair: (1) Impulses sometimes increased in frequency while the slow monophasic positive potential was induced. (2) In some cases a chemical stimulus gave rise to a slow diphasic potential; even at a phase near the peak of the positivity impulse frequencies were as high as at the peak of the negativity. These characteristics might result from the existence of a large number of chemosensory units in the single sensillum on the antenna, and could be explained assuming a complicated combination of a hyperpolarization followed by an after-negativity and a depolarization followed by an after-positivity. The after-positivity was not detected in the labellar chemoreceptor, and the high level of spontaneous activities in the sensilla basiconica might be responsible for it.

SUMMARY

1. The electrical responses of the sensilla basiconica on the antennae of the silkworm larva, *Bombyx mori*, have been studied by the microcapillary electrode technique.

2. Olfactory stimulation generally evokes a slow negative potential accompanied by increase in impulse frequency; but some agents evoke a slow diphasic potential, from negative to positive.

3. Strong vapours of anaesthetics evoke a slow positive potential with decrease in impulse frequency.

4. By comparison with the case of the labellar chemosensory hair of the blowfly, these results on the silkworm are explained in terms of depolarization or hyperpolarization of the receptor membrane.

We wish to express our cordial thanks to Prof. M. Kuwabara and to Mr H. Tateda for valuable discussions and criticisms. We should like to thank Dr H. Chikushi (Laboratory of Sericology, Kyushu University) for supplying the silkworm larvae, Dr T. Watanabe (Department of Agricultural Chemistry, Kyushu University) for supplying some chemicals and Miss K. Shigetomi for her assistance in preparation of this manuscript. The present study was aided in part by the Scientific Research Fund from the Ministry of Education of Japan. Contributions from the Department of Biology, Faculty of Science, Kyushu University, no. 107.

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